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Sylvia B. Smith
Tsung-Ping Su *Editors*

Sigma Receptors: Their Role in Disease and as Therapeutic Targets

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Sigma Receptors: Their Role in Disease and as Therapeutic Targets

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Preface

In 2007, a book entitled *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications* was published by Springer (Neuroscience Division) with Drs. R. Matsumoto, W. Bowen, and T. Su as editors. Since that time, the field of study regarding sigma receptors has exploded. Indeed in 2006 (at the time the last book would have been updated), there were ~1200 papers published about sigma receptor; by late 2016, there were more than 4000 publications. The significant progress in the field necessitated a compendium focused on the role of sigma receptors in disease and their potential role as therapeutic targets.

Originally confused with opioid receptors and then orphan receptors with no biological function, sigma-1 receptor is now recognized as relevant to many degenerative diseases with remarkable potential as a therapeutic target. In this text, new information about the crystal structure of sigma-1 receptor and its binding sites are provided as well as its expression in many cell types. Its putative role in degenerative neuronal diseases including amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, pain, drug addiction, and locomotor activity is described. Its role in cancer biology and its promising potential in treatment of blinding visual diseases emphasize the tremendous far-reaching potential for ligands for these receptors. There has been progress in our understanding of sigma-2 receptor, which is covered in this text as well.

Exciting breakthroughs in the dynamic field of sigma receptor biology in the last decade are reported herein, which we hope will guide future investigators in determining the full potential of this unique, yet abundantly, expressed protein. We thank the many investigators who contributed to this work and look forward to continuing discoveries as the field of sigma receptor biology unfolds.

We are grateful to Dr. Meran Owen, Senior Publishing Editor at Springer, for reaching out to us and suggesting the need for this updated volume. We appreciate the efforts of Tanja Koppejan, who oversaw the publishing of this work. We acknowledge with gratitude the capable support of Mrs. Heide Andrews for her assistance in editing the volume.

Augusta, GA, USA — Sylvia B. Smith
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1 Introduction to Sigma Receptors: Their Role in Disease and as Therapeutic Targets

Sylvia B. Smith

Abstract

This book highlights contributions from leaders in the field of sigma receptor research. Sigma receptors represent a promising, novel target for the treatment of neurodegenerative diseases, retinal degenerations, pain and substance abuse. Information is presented about tracers for molecular imaging these receptors, the newly determined crystal structure of human sigma 1 receptor and information about sigma 2 receptor. New discoveries about the role of sigma 1 receptors in cancer, pain, neuropsychiatric disorders, learning and memory, neuronal networks and depression are described. The compendium offers important insights about the direction unfolding for this exciting field of research.

Keywords

Sigma receptor • Neuroprotection • Neuronal degeneration • Therapeutic target • Retinal degeneration • Alcoholism • Drug addiction • Substance abuse • Crystal structure sigma receptor • Amyotrophic lateral sclerosis • Pain

Forty years ago (in 1976), Martin and colleagues described the existence of several types of opioid receptors that mediated pharmacological effects of morphine and its structural analogues [1]. The opioid receptors were named using Greek symbols based on the first letter of the alphabet of the compound to which it bound. Thus, mu (μ) opioid receptors mediated *m*orphine-induced analgesia, kappa (κ) opioid receptors mediated *k*etocyclazocine-induced dysphoria and (σ) opioid receptors mediated *S*KF-10047 -induced psychotomimesis. Six years later, Su and colleagues demonstrated the existence of a "sigma receptor" that differed from the receptor identified by Martin, in that it had a low affinity for naltrexone, which is a high-affinity blocker for all opioid receptor subtypes [2]. This discovery led to iden-

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tification of a unique drug selectivity pattern that distinguished sigma receptors from other known opioid receptors.

The history regarding the early characterization of sigma receptors and confusion regarding their identification as opioid receptors is explained thoroughly in Dr. Rae Matsumoto‘s introductory chapter to the book *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications* (published by Springer in 2007). The reader is referred to that book comprised of an excellent collection of chapters by leaders in the field describing classes of sigma receptor ligands, cloning of sigma 1 receptor, and subcellular localization of the receptor [3]. The book notes a number of areas in which sigma receptor function is implicated including synaptic plasticity, modulation of ion channels, depression, drug abuse and gastrointestinal function.

Now, a decade later there is considerable interest in sigma receptors and their role in disease as well as their potential as therapeutic targets. In this text, which was invited by Springer Publishing Company, the focus is on the role of sigma receptor and its relation to disease. Owing to its location in the endoplasmic reticulum (ER), the ER-mitochondrial associated membrane, the plasma membrane and the nuclear membrane and reports that it interacts with a number of proteins, the suggestion has been put forth that sigma 1 receptor is a pluripotent modulator of cell survival [4].

In this text, we are pleased to highlight a number of advances in the field. Dr. Andrew Kruse and his team recently determined the first crystal structure of human sigma-1 receptor [5] and in Chap. 2, they offer detailed views of the sigma-1 architecture noting its very unusual folding in the membrane. This was a major breakthrough for the field. Dr. Jason Schnell and colleagues discuss this structure in Chap. 3 and the interesting questions related to molecular ‘tricks’ used by sigma-1 receptor to modulate myriad signaling events. Our understanding of sigma-1 receptors has been significantly enhanced by contributions from the Wünsch lab that uses fluorinated positron emission tomography traces to image sigma-1 receptor in the central nervous system. In Chap. 4 they provide detailed information on fluoroalkyl substituted spirocyclic PET tracers, which are the most promising tracers described to date.

While the emphasis of this book is on sigma-1 receptors, the field of sigma-2 receptors has experienced significant breakthroughs. Dr. Robert Mach and his team have studied sigma-2 receptors extensively and in Chap. 5, they describe the evolution of this protein from an obscure binding site to a therapeutic target. It is established that sigma-2 receptor is a biomarker of tumor cell proliferation.Indeed sigma-2 receptor agonists are potent anticancer agents. Balancing those findings, Chap. 6 contributed by Soriani’s group reviews how pro-survival functions of sigma-1 receptor can be hijacked by cancer cells to shape their electrical signature and behavior in response to the tumor microenvironment.

The emphasis of the remainder of the book is on sigma-1 receptor in disease, especially related to function. In Chap. 7, investigators Tsai and Su provide intriguing data about the role of sigma-1 receptor in axon guidance and in balancing the populations of neuron and glia and their implications in CNS diseases. This a major attribute of the nervous system. In Chap. 8, Vela and colleagues outline the role of pharmacological modulation of sigma-1 receptor to treat pain. Intriguingly, sigma-1 receptors are expressed abundantly in various pain centers of the central nervous system and modulate receptors and ion channels.The chapter describes in-depth several types of pain, including chronic pain, and modulation by sigma-1 receptor ligands. The issue of analgesia is addressed also by Cobos and coworkers in Chap. 9, but the focus is on antagonists of sigma-1 receptor. The group reviews interesting preclinical evidence that has led to the development of the first selective sigma-1 antagonist with an intended indication for pain treatment, which is currently in Phase II clinical trials.

Sigma receptor has been implicated in a number of neurodegenerative diseases and this very complex area of investigation is reviewed comprehensively by Dr. Matsumoto and her associates in Chap. 10. The intriguing action of ligands for sigma-1 receptors in modulating multiple

neurodegenerative processes, including excitotoxicity, calcium dysregulation, mitochondrial and ER dysfunction, inflammation, and astrogliosis is discussed along with the potential of these ligands in treating CNS disease. Outlined in this chapter is convincing evidence that sigma-1 receptor dysfunction worsens disease progression, whereas enhancement amplifies pre-existing functional mechanisms of neuroprotection and/or restoration to slow disease progression.

Related to neurodegenerative diseases is the field of neuropsyciatric disorders and Dr. Kenji Hashimoto has contributed Chap. 11, which describes the relationship of serotonin reuptake inhibitors and sigma-1 receptors. The chapter describes several compounds including fluvoxamine and ifenprodil, which may provide benefit to patients with neuropsychiatric disorders. Chap. 12 contributed by Dr. Shilpa Buch and her colleagues reviews sigma-1 receptor and neurodegenerative disease, with an emphasis on cocaine abuse. It is noteworthy that studies suggest that cocaine's interaction with sigma-1 receptor may be related to impairment of blood-brain barrier, microglial activation and astrogliosis. Complementing this contribution is Chap. 13, submitted by Dr. Valentina Sabino and her collaborators, examining a wide spectrum of drugs of abuse and their relationship to sigma-1 receptors. There is considerable evidence that sigma-1 receptors are involved in addictive and neurotoxic properties of abused drugs.

Work from Fukunaga's lab has examined the role of sigma-1 receptor in modulating depressive behaviors using mice. The work is described comprehensively in Chap. 14 and explores Akt signaling in hippocampus. Another area that has been investigated for some time is the role of sigma-1 receptor in learning and memory.Dr. Tangui Maurice, a leader in this field of study, describes in Chap. 15 that sigma-1 receptor agonists have anti-amnesic properties due to mobilization of calcium and modulating of glutamate and acetylcholine systems. Indeed, new studies suggest that sigma-1 receptor is a potential target in treatment of Alzheimer's disease.The role of sigma-1 receptor in neurodegenerative diseases have been investigated also with respect to motoneuron diseases.In Chaps. 16 and 17, the role of sigma-1 receptors in amyotrophic lateral sclerosis (ALS).This devastating neurodegenerative disease affects spinal cord and brain motoneurons leading to paralysis and early death. Indeed, mutations in sigma-1 receptor have been described in familiar ALS.These chapters contributed by Navarro's group and Ruoho's team describe promising studies about the potential of targeting sigma-1 receptor in ALS treatment.

The final three chapters of this book focus on sigma-1 receptor and the retina, which has been an active area of research for the past two decades. Sigma-1 receptor is highly expressed in retina and it is clear that this receptor has powerful neuroprotective properties in this tissue. Chap. 18 is a contribution from our lab. It provides an overview of retinal architecture and reviews early work in the field related to this tissue. It also describes extremely exciting *in vivo* findings of the potential of targeting sigma-1 receptor in diabetic retinopathy and in severe photoreceptor degeneration. Chap. 19 contributed by Drs. Mavlyutov and Guo describes sigma-1 receptor as an endogenous neuroprotective mechanism in the retina. They describe important findings from their laboratory about the location of sigma-1 receptor on the nuclear envelope of various retinal cell types including ganglion and photoreceptor cells. The final Chap. 20 discusses the potential of targeting sigma-1 receptor in glaucoma, the second leading cause of blindness worldwide. Dr. Kathryn Bollinger is a glaucoma specialist who manages this challenging disease in humans and is attempting to preserve function of ganglion cells using sigma-1 receptor ligands in rodent models of glaucoma.

In summary, the chapters described herein provide information about the structure and function of sigma-1 receptor and to a lesser extent sigma-2 receptor. Studies from numerous investigators suggest a broad spectrum of disorders and diseases that are impacted by the field of sigma receptor biology. The apparent ability of sigma-1 receptors in modulating many key cellular functions and the potential to target this receptor in treating debilitating diseases is both intriguing and compelling. It is hoped that the information

conveyed in this compendium will capture the interest of newcomers to the field! We await the next breakthroughs in this extremely exciting area of cell and molecular biology.

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Structural Perspectives on Sigma-1 Receptor Function

2

Assaf Alon, Hayden Schmidt, Sanduo Zheng, and Andrew C. Kruse

Abstract

The sigma-1 receptor is an enigmatic ER-resident transmembrane protein linked to a variety of human diseases. Although the receptor was first cloned 20 years ago, the molecular structure of the protein and the mechanistic basis for its interaction with drug-like small molecules have remained unclear until recently. The determination of the first crystal structure of human sigma-1 offered the first detailed views of the sigma-1 architecture, and revealed an unusual overall fold with a single transmembrane helix in each protomer. The structure shows an overall trimeric receptor arrangement, and each protomer binds a single ligand molecule at the center of its carboxy-terminal domain. These results offer detailed molecular views of receptor structure, oligomerization, and ligand recognition, providing a framework for the next era of sigma-1 research.

Keywords

Sigma-1 receptor • Structural biology • Crystallography • Lipidic cubic phase • Membrane protein

2.1 Introduction

The sigma-1 receptor is an unusual transmembrane protein implicated in a broad range of cellular functions and with possible roles in both normal and disease states in humans [1]. Since its discovery decades ago, the sigma-1 receptor has been implicated in a diverse array of pathophysiological conditions ranging from neurodegenerative disease [2] to cancer [3], and it has been reported to interact with numerous proteins including chaperones [4], ion channels [5, 6], and

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GPCRs [7]. Like the true opioid receptors, sigma-1 shows high affinity for benzomorphan compounds and on this basis it was originally classified as a member of this family [8]. However, subsequent studies with enantiomerically pure probe compounds showed that sigma-1 exhibits a preference for (+) benzomorphans, while true opioid receptors bind with high affinity only to the (−) enantiomer [9]. The endogenous ligand of sigma-1, if any, remains unclear. Although the hallucinogen N,N-dimethyl tryptamine (DMT) has been reported as a possible ligand [10], a subsequent study has cast doubt on this idea [11].

Although the pharmacology and cell biology of sigma-1 has been extensively studied for decades, it was not until 1996 that the first information regarding the molecular architecture of the protein became available when the receptor was cloned. Sigma-1 was first cloned from guinea pig [12], using classical biochemical techniques to isolate the receptor by tracking binding activity and then using degenerate oligonucleotide probes to clone the receptor for a cDNA library. The receptor was subsequently cloned from a human placental choriocarcinoma cDNA library [13], as well as from mouse [14] and rat [15] tissues.

The amino acid sequence of the receptor showed no similarity to any other mammalian protein, although it resembled that of the fungal sterol isomerase Erg2p. Hydrophobicity analysis of the sequence showed a highly hydrophobic segment at the receptor amino terminus, predicted to be a transmembrane domain. Initially it was proposed that this was the sole transmembrane helix in the receptor [12], although later a two-pass transmembrane model came to be more widely embraced. The latter model was supported primarily by a report of immunostaining experiments with antibodies to green fluorescent protein (GFP) fused to either the amino- or carboxy-terminus of the receptor [16]. An important caveat, however, is the fact that GFP is often poorly secreted, and the fusion protein may have exhibited aberrant membrane insertion properties. Nonetheless, the two-pass transmembrane model was widely embraced, and served as the basis for molecular modeling studies [17] and efforts to map the putative second transmembrane helix [18]. As discussed below, however, the crystal structure of the receptor shows only a single transmembrane domain, consistent with the earliest structural models rather than those that followed.

2.2 Approach to Structure Determination

Recent advances in membrane protein structural biology have revolutionized structure determination for human membrane proteins [19], particularly G protein-coupled receptors (GPCRs). Key advances include the widespread use of lipid-based crystallization methods [20] and concomitant improvements in X-ray diffraction methods for microcrystals [21]. Taken together, these techniques allow crystallization of membrane proteins in a lipid bilayer system similar to their biological milieu, improving the stability of the proteins and allowing examination of their structural interactions with lipids. Other important advances including the use of new detergents [22] have also had a major impact on membrane protein biochemistry, allowing straightforward manipulation of otherwise intractable receptors.

In approaching structural analysis of the sigma-1 receptor, a GPCR-inspired approach was used. While previous methods for sigma-1 biochemistry involved bacterial expression [23] and the use of harsh detergents like Triton X-100, our crystallization effort focused instead on expression in eukaryotic cells and purification in milder maltoside detergents. In brief, this entailed use of *Sf9* insect cells and baculovirus transduction to produce receptor at high levels, followed by extraction in detergent and purification by antibody affinity chromatography [24]. This approach yielded pure and almost monodisperse receptor with minimal modifications to the receptor sequence. Following proteolitic removal of the amino-terminal FLAG epitope tag, the resulting crystallization sample contained only a four amino amino acid modification, “GPGS”, at the receptor’s amino terminus, with all other parts matching the wildtype human sigma-1 sequence.

Following purification, crystallization of the receptor was straightforward using the lipidic cubic phase technique, and with optimization crystallographic datasets were obtained for sigma-1 bound to PD144418, a high affinity antagonist [25], and the compound 4-IBP, which has an incompletely understood efficacy profile [26]. Structure determination was hindered by the lack of related structures for phase calculation, but after an extensive screening campaign a suitable dataset was obtained by soaking with tantalum bromide clusters [27], allowing SIRAS phase calculation [24].

2.3 Overall Structure of Sigma-1

The crystal structures of the human sigma-1 receptor revealed an unusual fold, unique among known protein structures, and confirmed a single-pass transmembrane topology, in contrast to most previous models [24]. The protein crystallizes as an intimately associated triangular trimer with a transmembrane domain at each corner (Fig. 2.1). Residues 6–31 comprise the single transmembrane helix, with residues 32–223 forming a carboxy-terminal/cytosolic domain consisting of a β-barrel (residues 81–176) and flanking α-helices. This β-barrel constitutes both the ligand-binding site and the oligomerization interface. An unusual and striking feature of the structure is the presence of two α-helices (residues 177–223), which cover the membrane-proximal opening of the β-barrel. These helices have hydrophobic amino acids pointing toward the membrane surface, suggesting that the membrane-adjacent face of the trimer may be embedded within the lipid bilayer (Fig. 2.2). In addition, three arginine residues on the outer helix of each protomer are positioned in a way that would allow their positively charged side chains to interact with the negatively charged phospholipid head groups present in the cell membrane. In the crystal, these residues interact with sulfate ions in the crystallization buffer. Ordered monoolein lipids are also resolved, defining the boundary of the membrane plane. Thus, the crystal structure suggests that while the sigma-1 receptor only has one transmembrane domain, the membrane-proximal region of the protein formed by these two helices is partially embedded in the cytosolic side of the ER membrane, allowing this surface to dock against the lipid bilayer.

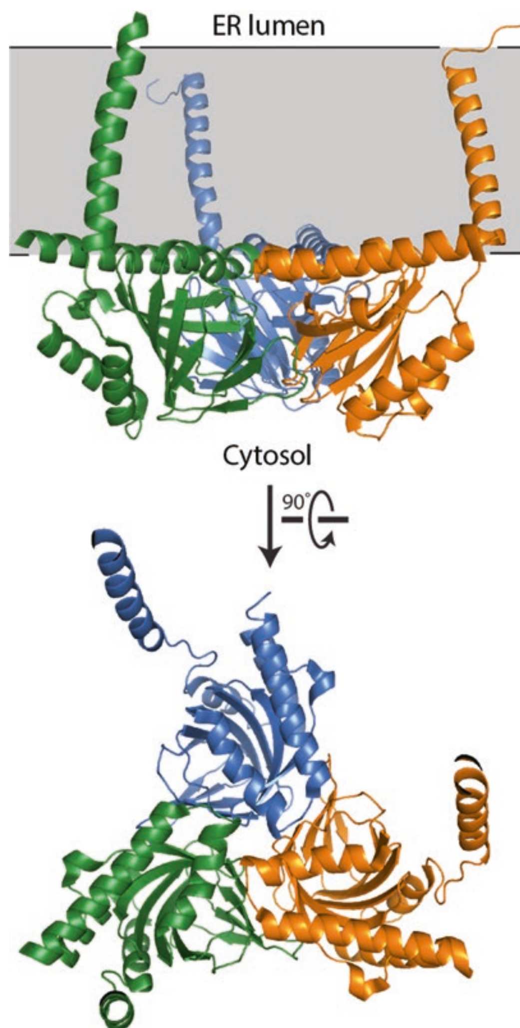

Fig. 2.1 The overall structure of the human sigma-1 receptor. From the side, the receptor is observed to sit with the membrane-proximal surface partially embedded in the membrane, which is depicted in *grey*. The membrane boundary was determined using the PPM prediction server [42]. Viewing the receptor normal to the membrane from the ER surface shows the trimeric arrangement and overall architecture of the sigma-1 receptor

Despite the unusual structure of the sigma-1 receptor as a whole, the β-barrel region bears

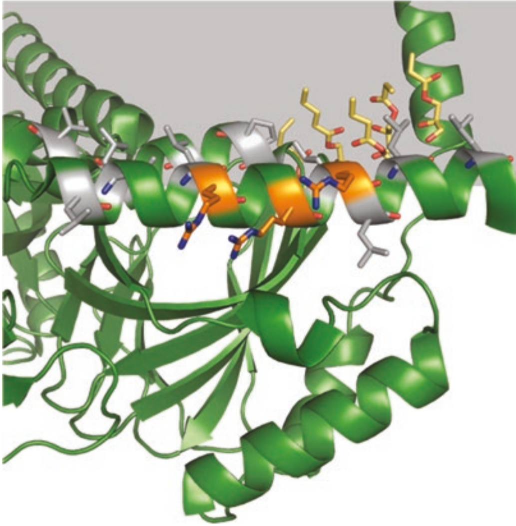

Fig. 2.2 The membrane-proximal region of the sigma-1 receptor. A close-up view of a single protomer shows lipids observed in the crystal structure (depicted as *yellow* sticks; most of the lipid tails are disordered and not resolved). *Shading* indicates the location of the membrane. Arginine residues (*orange*) are well positioned to interact with phospholipid headgroups in the membrane, and many of the hydrophobic residues in the membrane-proximal helices (*grey*) are positioned in such a way as to be embedded in the hydrophobic membrane interior

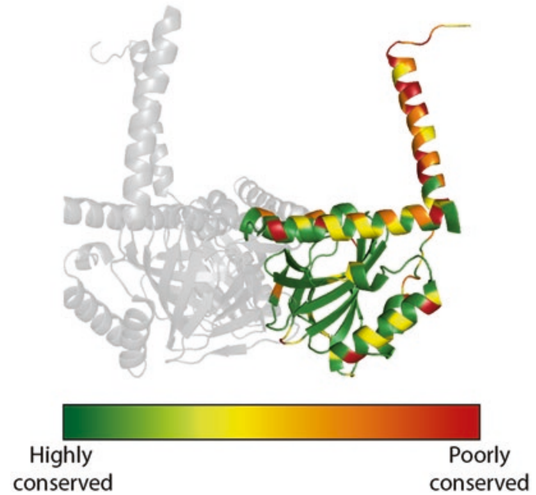

Fig. 2.3 Conservation of the sigma-1 receptor protomer. The receptor is shown with highly conserved regions colored in *green* and poorly conserved regions colored in *red*. Conservation analysis was performed using the ConSurf web server [43], using the most similar 300 sequences to the human sigma-1 receptor. The conservation map shows that the β-barrel region including the ligand-binding site and oligomerization interface is highly conserved, while the transmembrane domain is relatively poorly conserved

significant structural similarity to cupin family proteins, with a root mean square deviation of 2.4–3.0 Å for most cupin domains [24], most of which are bacterial enzymes that also exhibit oligomerization. While there is no evidence of direct functional similarities between these proteins and sigma-1 receptor, the ligand-binding site of sigma-1 appears to be analogous to the active site of these proteins, which may suggest that the sigma-1 receptor descended from an enzyme that was evolutionarily repurposed. Indeed, the sigma-1 receptor's closest homolog of well described function is the yeast sterol isomerase Erg2p [12].

Conservation analysis provides clues to the functional importance of the different regions of the sigma-1 receptor. The transmembrane helix is rather poorly conserved, with a relatively high degree of variation in the sequence among sigma-1 homologs (Fig. 2.3). The only sequence constraint on this helix appears to be the need for hydrophobicity, which suggests that the transmembrane helix is primarily an anchor to the membrane with little other function. In contrast, the β-barrel region, which includes the ligand-binding site and oligomerization interface, is almost perfectly conserved (Fig. 2.3). This suggests that both the ligand-binding site and the oligomerization interface are integral to sigma-1 receptor function.

2.4 Oligomerization

In the crystal structure, sigma-1 is arranged as a triangular trimer with a ligand binding site in each protomer. Each interface between protomers buries roughly 9300 Å [2] of surface area, and the homotrimer interface is highly conserved across different species suggesting trimerization is physiologically relevant and not merely due to crystal packing (Fig. 2.4). The interface comprises a mix of polar and hydrophobic contacts. In particular, GxxxG motif (G87–G91), which was proposed to be part of a putative second

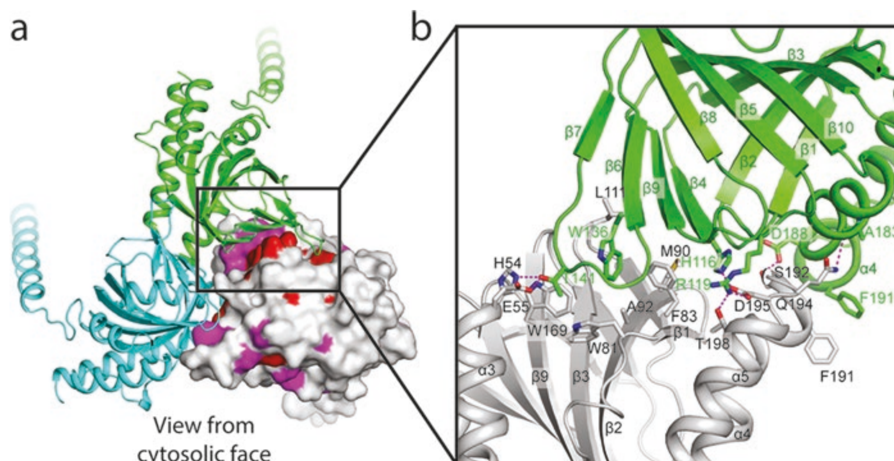

Fig. 2.4 Oligomerization interface. (**a**) One protomer is shown as a surface and colored by sequence conservation, with residues more than 98 % and 80 % conservation highlighted in *red* and *magenta*, respectively. (**b**) A detailed view of interface with residues shown as sticks. The *dashed lines* indicate hydrogen bond and salt bridge interactions

transmembrane domain [16, 18], actually forms a beta-hairpin structure in the cytosolic domain buried deeply inside the center of interface and required for oligomerization and ligand binding [28]. From structural view, the distance between Cα atoms of G88 in each protomer is about 6 Å, consequently mutation of glycine to a large side chain residue would introduce a clash inside the interface, accounting for the observation that the G88L mutation favors the monomeric state over higher oligomeric states. Interestingly, the G88L mutant also exhibited a significant decrease in ligand binding, suggesting that the GxxxG-mediated oligomerization is likely important in either binding or protein folding, since G88 is distant from ligand binding site [28]. The correlation between oligomerization and ligand binding was also supported by the observation that oligomeric sigma-1 retained ligand binding while monomeric forms lost binding ability [28].

However, despite the availability of the crystal structure of sigma-1 in a trimeric form, its oligomerization state *in vivo* remains uncertain. Detergent solubilized human sigma-1 in the presence of antagonist showed a broad range of oligomerization states as revealed by size-exclusion chromatography with multi-angle light scattering (SEC-MALS) as well as Native PAGE analysis [24]. In addition, several high molecular weight bands corresponding to tetramer and pentamer were identified using sigma-1 in rat liver membrane photoaffinity labeled by a radioiodinated ligand [29]. Taken together, these data suggest the sigma-1 trimer observed crystallographically may represent only one of many diverse oligomerization states existing *in vivo* which is prone to crystallization.

A related and important question regards the relationship between oligomerization and receptor activation. How do agonists and antagonists induce distinct cellular effects through the sigma-1 receptor? A cell-based study using FRET approaches revealed that in the absence of ligand, sigma-1 existed as a combination of different oligomeric states, while antagonist stabilized higher order oligomer, agonist instead favored small oligomers [30]. However, little difference in oligomerization was observed among ligand-free, agonist and antagonist bound sigma-1 when solubilized in detergent [24]. However, detergents do not perfectly mimic native membrane environments, thus these conditions may not reflect the actual state *in vivo*. A full understanding of sigma-1 oligomerization will require further biophysical and structural studies, including techniques like cryo-electron microscopy and NMR using sigma-1 reconstituted in lipid bilayers.

2.5 Ligand Recognition

The sigma-1 receptor has been shown to bind with high affinity and specificity to a variety of structurally diverse compounds [8]. Numerous structure-activity relationship (SAR) studies have been performed in an attempt to develop a common pharmacophore model, but the only common features shared by virtually all high-affinity sigma-1 ligands are a cationic amine and at least one aromatic ring, typically with three intervening methylenes [31]. Both sigma-1 crystal structures include bound ligands, offering a structural view of the sigma-1 receptor's unique pharmacology.

The binding pocket of sigma-1 receptor is located in the center of a cone-shaped β-barrel that is gated at its wider side by two hydrophobic, membrane-parallel helices as discussed above (Fig. 2.1). In the crystal structure, the binding pocket is completely occluded from the solvent, and it remains unclear how ligands access the active site. The two possible pathways are either from the membrane through the two gating membrane-adjacent helices, or from the cytoplasm, through the narrow polar opening obstructed primarily by Gln135.

The sigma-1 receptor binding site is a wide and oblong cavity in the heart of the cytoplasmic domain. The binding pocket is lined with aromatic and hydrophobic residues, mirroring the hydrophobic nature of typical sigma-1 ligands. The only exceptions to the general hydrophobic character are the acidic residues Glu172 and Asp126. The former is highly conserved, and mutations in this position completely abrogate ligand binding as it serves to coordinate the positive charge of the ligand's cationic amine [32]. Asp126, probably in a protonated form, and Tyr103 are positioned to stabilize and fix the orientation of Glu172. The relative scarcity of polar residues in the binding site and the many flexible hydrophobic residues such as leucine and methionine likely contribute to the pharmacological promiscuity of the receptor.

Two structures of sigma-1 receptor were solved, one of the receptor bound to PD144418, a sigma-1 antagonist [25], and another with the receptor bound to 4-IBP, a high-affinity ligand with a poorly characterized efficacy profile [26, 33]. These two compounds are chemically divergent, sharing only an elongated shape and a central cationic amine. Despite this, they bind to sigma-1 in a very similar manner (Fig. 2.5). The binding mode of these molecules is in agreement with phamacophore models of sigma-1 that predicted two hydrophobic sites on both sides of the

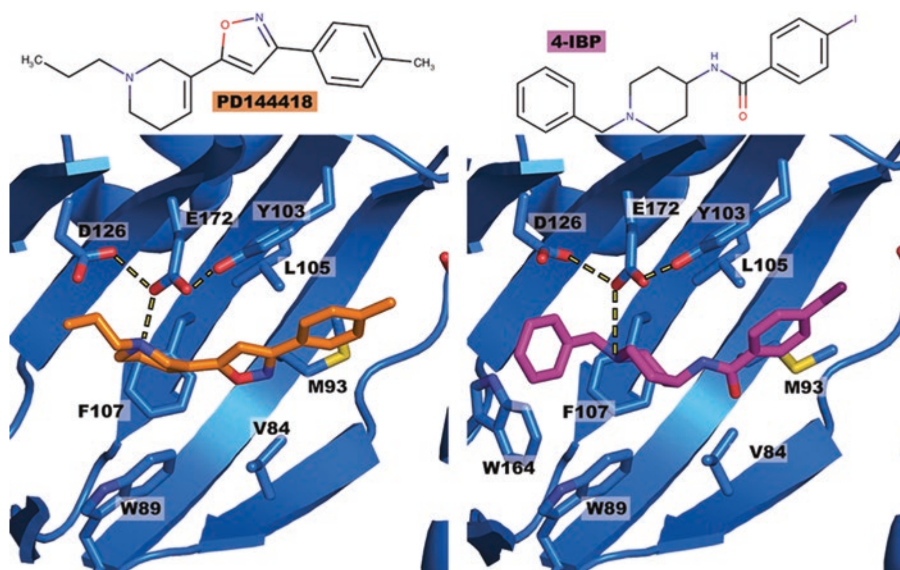

Fig. 2.5 Ligand recognition by sigma-1. The structures of sigma-1 bound to PD144418 (*left*) and 4-IBP (*right*) are shown. The interactions with the ligand in each case are predominantly hydrophobic in character, with the exception of a salt bridge interaction between the ligand amine and receptor Glu172. The two compounds bind with very similar overall poses, despite only modest chemical similarity

cationic amine [31, 34], a primary site 6–10 Å from the cationic amine and a secondary site at a distance of 2.5–4 Å. The binding pocket also contains two tryptophan residues (Trp89 and Trp164), offering an explanation for the observed attenuation in binding upon exposure of sigma-1 to UV radiation [35].

2.6 Disease-Associated Mutations

A number of mutations in sigma-1 have been linked to neurodegenerative disease in humans. Some of these occur in untranslated regions and may affect protein abundance [36, 37], while other mutations occur in the protein coding sequence [38–40]. For the latter class, the availability of structural information now offers new insight into the molecular mechanisms of sigma-1 dysfunction (Fig. 2.6).

One mutation, E102Q, was identified in a consanguineous family in Saudi Arabia and causes a juvenile-onset ALS-like neurodegenerative disease [39]. This mutation was subsequently shown to alter sigma-1 localization and mobility in cells [41]. Glu102 is highly conserved residue, and the crystal structure reveals an unusual role as a double hydrogen bond acceptor to Val36 and Phe37 backbone amines (Fig. 2.6a, b). Mutation of this to glutamine would disrupt one of these interactions, resulting in an unfavorable apposition of

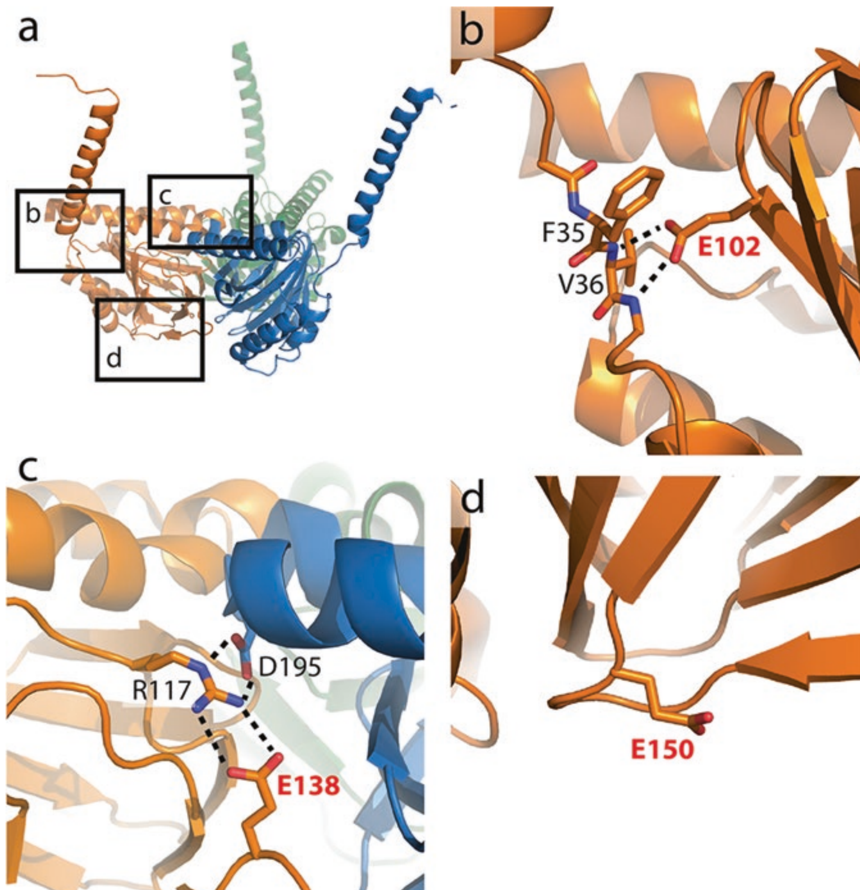

Fig. 2.6 **Disease-associated mutations in sigma-1**. (**a**) Overall view of the receptor with sites for human disease-associated point mutations in *labeled boxes*. (**b**) E102Q mutation associated with ALS likely disrupts hydrogen bond network. (**c**) E138Q mutation similarly prevents formation of salt bridge and hydrogen bond network with R117, which links receptor protomers together. (**d**) The surface-exposed E150K mutation is more enigmatic, with no clear reason for structural disruption upon mutation

two hydrogen bond donors. A second mutation, E138Q, shows a similar hydrogen bonding network (Fig. 2.6c), and likewise is associated with autosomal-recessive distal hereditary motor neuropathy [40].

A third mutation in the coding sequence, E150K, is associated with a similar hereditary motor dysfunction [40]. Unlike E102Q and E138Q however, the molecular basis for the effect of this mutation remains unclear. Glu150 is a surface-exposed residue interacting largely with solvent, and mutation to lysine is unlikely to significantly alter receptor folding (Fig. 2.6d). Instead, this residue may play a role in sigma-1 interaction with effector proteins, or in some other as yet uncharacterized process.

2.7 Outlook

With the availability of high quality structural information, our understanding of sigma-1 function is poised for transformation. The detailed views of the ligand binding site will allow rational design of new sigma-1 ligands, with potentially unexpected properties, and the overall structure will enable rational design of engineered receptor constructs. However, many other important questions remain. The molecular distinction between agonists and antagonists, as well as the mechanisms of receptor activation are likely to be particularly important areas for understanding the molecular basis of sigma-1 function in years to come. In addition, a full understanding of sigma-1 activity will require studies of the receptor in complex with effector proteins, as well as further investigation of the role of oligomerization and its potential regulation by small molecule compounds.

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3 A Review of the Human Sigma-1 Receptor Structure

Felipe Ossa, Jason R. Schnell,
and José Luis Ortega-Roldan

Abstract

The Sigma-1 Receptor (S1R) is a small, ligand-regulated integral membrane protein involved in cell homeostasis and the cellular stress response. The receptor has a multitude of protein and small molecule interaction partners with therapeutic potential. Newly reported structures of the human S1R in ligand-bound states provides essential insights into small molecule binding in the context of the overall protein structure. The structure also raises many interesting questions and provides an excellent starting point for understanding the molecular tricks employed by this small membrane receptor to modulate a large number of signaling events. Here, we review insights from the structures of ligand-bound S1R in the context of previous biochemical studies and propose, from a structural viewpoint, a set of important future directions.

Keywords

Sigma-1 receptor • Protein structure • Membrane protein • Ligand binding • Protein oligomerization

3.1 Introduction

The Sigma-1 Receptor (S1R) presents an extremely compelling cell-biological and structural puzzle. Despite its small size S1R acts to regulate the activity of a large number of cellular proteins and is itself regulated by small molecule binding [1]. The reported protein interaction partners include ion channels (both ligand and voltage gated), GPCRs, transcription factors and the endoplasmic reticulum (ER) chaperone protein BiP [2]. Among the large number of small mole-

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cules that S1R binds to are cocaine [3, 4] (a stimulant and drug of abuse), haloperidol [5] (an antipsychotic), fluvoxamine [6] (an antidepressant), steroid hormones such as progesterone [7], and single-chain lipid-like compounds such as sphingosine [8] and myristic acid [9]. Although S1R is thought to reside primarily in the mitochondria associated membrane (MAM) of the ER [2], it has been reported to relocalize to the plasma membrane and the nuclear membrane [10–13]. The physiological processes that S1R is involved in include neurotransmission, regulation of intracellular calcium concentrations, and cell survival. S1R dysfunction has been implicated in neurological disorders such as depression and addiction [14], and neuropathic pain [15].

3.2 Overview of S1R Sequence and Structure

The existence of S1R has been known from pharmacological and radio-ligand binding studies for ~40 years [5, 16], although the protein itself was not discovered for another 20 years [17]. Based on its amino acid sequence, S1R is a member of the *ERG2* family of membrane proteins, having approximately 33 % identity and 66 % similarity to the fungal $\Delta 8 \rightarrow \Delta 7$ sterol isomerases [17, 18] (Fig. 3.1). Now, another 20 years after the identification of the amino acid sequence, the first structure of full-length human S1R has been reported [19]. Two trimeric structures of the receptor bound to different small molecule ligands were determined (Fig. 3.2a). One ligand, PD144418, is a known antagonist.

The S1R subunit structure has at its center two anti-parallel beta-sheets that form a squashed barrel-like structure (Fig. 3.2b). The arrangement of the beta-sheets places it within the cupin fold family, also known as the jelly-roll family. The cupin fold is functionally versatile with members including metalloenzymes from several enzyme classes as well as seed storage proteins [20]. Metalloenzymes with the cupin fold have been observed to use Fe, Mn, Ni, Zn, Co, and Cu [20], and include the oxidoreductases thiol dioxygenase [21] and 2-oxoglutarate oxygenase [22], the lyase ectoine synthase, and the hydrolase KdgF [23]. The yeast sterol isomerases that are most similar in sequence to S1R (see below) have a modest affinity to Zn^{+2}, but S1R does not bind metals with high affinity [24], nor does it exhibit isomerase activity [17].

The strands of the two sheets in the cupin fold of S1R are rotated at a ~30–40° angle to each other. The two sheets of S1R make few noncovalent contacts, with the most conspicuous being a hydrogen bond between the backbone amide of Met90 in the highly curved strand 2 of the larger sheet to the sidechain hydroxyl of Ser113 in strand 4 in the smaller sheet. In the *ERG2* family, only serine or threonine are found at position 113 suggesting that this may be an important contact for structural stability (Fig. 3.1).

The β-sheets of S1R are flanked on both the N- and C-termini by helical regions (Fig. 3.2b, c). The N-terminus contains the transmembrane helix (residues ~8–32), which is followed by two helices (denoted A and B) that form a helical hairpin that lies on the surface of the larger 6-stranded sheet. The C-terminal region contains two helices (denoted D and E), which form a flat, hydrophobic surface that likely interacts with membrane [19]. In solution nuclear magnetic resonance (NMR) studies of an N-terminally truncated S1R construct the residues ~183–189 and 197–204 were shown to interact with detergent acyl chains [25]. These residues are juxtaposed in the receptor and are probably strongly associated with the membrane (Fig. 3.2d).

3.3 The S1R Ligand Binding Pocket and Pharmacophore

In the receptor structures the ligands bind at a similar position within the β-sheets, and the binding site is analogous to the active site of the cupin fold metalloenzymes. Ligands bind to protein with a 1:1 stoichiometry and each ligand contacts only a single subunit within the trimer. The ligands bind in extended conformations and are surrounded by protein on all sides. The binding site is largely consistent with earlier sequence

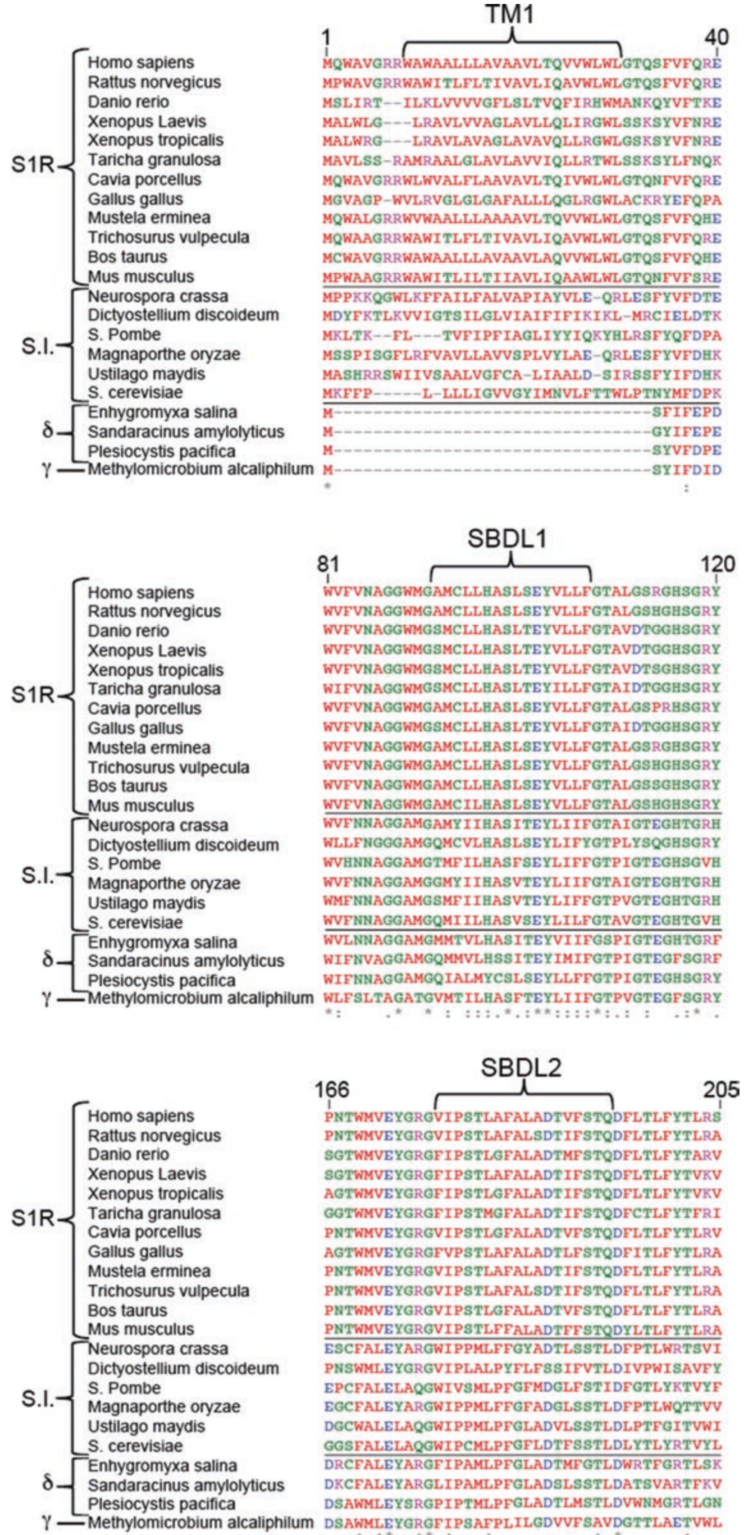

Fig. 3.1 Local sequence alignments of the *ERG2* family. Selected S1R sequences are aligned with the fungal Δ8 → Δ7 sterol isomerases and four related sequences from γ- or δ-proteobacteria for the TM1 region (*top*; residues 1–40 of human S1R), the SBDL1 region (*middle*; residues 81–120 of human S1R), and the SBDL2 region (residues 176–205 of human S1R). The local alignments were extracted from an alignment of the full-length sequences carried out with T-Coffee [73]. For the TM1 region, the unconserved inserts of residues 7–41, 8–23 and 6–20 of *N. crassa*, *D. discoideum*, and *U. maydis*, respectively, were removed prior to aligning. Below each alignment is indicated whether the position is strictly conserved (*), conserved as amino acids of strongly similar properties (:), or conserved as amino acids of weakly similar properties (.). Amino acid names are colored according to whether they are nonpolar (*red*), polar uncharged (*green*), polar positively charged (*magenta*), or polar negatively charged (*blue*)

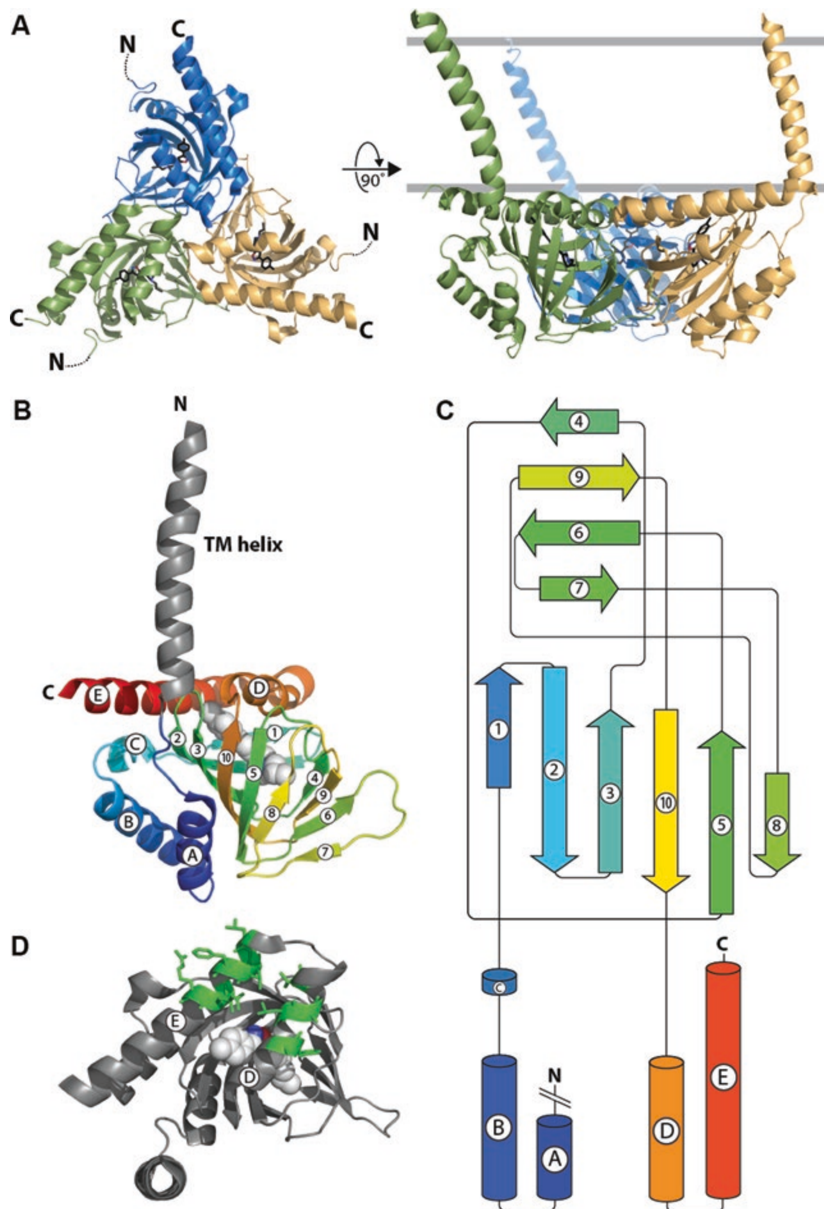

Fig. 3.2 Structural overview and secondary structure topology of human S1R. (**a**) Cartoon diagram of the homotrimeric structure of S1R (PDB: 5HK1). *Left*: view down the symmetry axis from the perspective of the membrane. For clarity, the transmembrane helices have been removed. *Right*: side view from within the membrane (shown as two, *gray*, horizontal lines). The ligand PD144418 is represented as *black* sticks. (**b**) Cartoon diagram of a single subunit bound to the ligand PD144418 (*white* van der Waals spheres) (PDB: 5HK1). The transmembrane helix is colored *gray*, and the rest of the cartoon is colored from *blue* (N-terminal) to *red* (C-terminal). Strand numbering and helix lettering are identical to that of the topology diagram. (**c**) Topology diagram illustrating the S1R fold in which two sheets are flanked on each end by helical regions. The two sheets are connected by a hydrogen bond between the backbone amide of Met90 at the N-terminus of strand 2 and the sidechain hydroxyl of Ser113 at the N-terminus of strand 4. The topology diagram was adapted from a Pro-Origami output [74]. (**d**) Cartoon diagram of S1R from the perspective of the membrane in which the TM helix is oriented toward the viewer. Residues 183–184, 186–187 and 189 in helix D and residues 197–200 and 202–204 in helix E that have been shown to interact strongly with detergent [25] are shown as sticks and shaded *green*. All structural figures were generated using PyMol (Schrödinger, LLC)

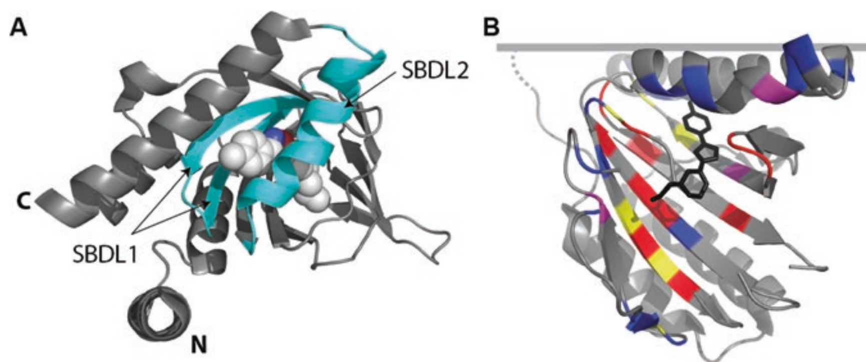

Fig. 3.3 (**a**) Cartoon diagram from the perspective of the membrane in which the TM helix is oriented toward the viewer. The SBDL regions 1 (residues 91–109) and 2 (residues 176–194) that were predicted from sequence analysis to be important in ligand binding [26, 27] are shaded in cyan. (**b**) Mapping of positions for which information on drug binding has been tested onto a subunit from PDB 5HK1 (Table 3.1). Positions at which substitutions have been shown to have large, moderate, or no significant effect on ligand binding are colored *red*, *yellow*, or *blue*, respectively. The positions of residues C94, H154, and D188, which have been shown to be derivatised by photoreactive probes are colored *magenta*. For clarity, strands 4, 6, and 9 of the small β-sheet have been removed. The ligand, PD144418, is represented as *black* sticks. The predicted position of the membrane is indicated by a *gray* line

analysis: because S1R ligands are able to bind to the ERG2 proteins in yeast and inhibit sterol isomerase activity, it was predicted that the two regions of highest amino acid conservation between S1R and the ERG2 would be involved in ligand binding [26]. These regions were later named the steroid binding domain-like (SBDL) 1 (residues 91–109) and 2 (residues 176–194) [27] (Fig. 3.1), and the importance of these regions in ligand binding was supported by derivatization studies using photoreactive analogs of cocaine [27] and fenpropimorph [28]. Chemical cross-linking and radio-ligand transfer between amino acids in SBDL1 and SBDL2 elegantly showed that the two regions are positioned close to each other in the folded receptor [28, 29]. In a satisfying confirmation of those predictions, the S1R structures show that SBDL1 and SBDL2 envelope the bound ligands (Fig. 3.3a) and account for the majority of the residues responsible for the primary hydrophobic site of the ligand pharmacophore (see below). SBDL1 consists of a β-hairpin (strands 2 and 3) within the larger of the two sheets and SBDL2 is a helix (helix D) that lies on top of the ligand binding site and also forms part of the membrane attachment region along with helix E.

Before the structure of S1R was known, a large number of studies reporting mutations, deletions, and labelling with photoreactive probes were reported (Table 3.1). The results of mutational studies are strongly supportive of the structural model, with most of the substitutions having large effects on ligand affinity found close to the ligand binding site (Fig. 3.3b). The ligand pharmacophore includes a positive ionizable feature, which is frequently a basic amine. This can now be seen in the structures to interact directly with E172 in the binding pocket. In turn, E172 is stabilized by an interaction with D126 and Y103. A role for residues E172 and D126 in ligand binding was discovered by Seth et al. [11], who tested glycine substitutions for each acidic residue in the C-terminal half of S1R for ligand binding. Substitution for either D126 or E172 abrogated drug binding [11], and later studies suggested that E172 was especially important, as even a conservative substitution for aspartic acid at this position abolished ligand binding [30]. The importance of the hydroxyl of Y103 was tested with a phenylalanine substitution in an early study of ligand binding mutants and resulted in reduced affinity for the agonist (+)-pentazocine and the antagonist NE-100 [31].

Table 3.1 Effects of reported mutations of S1R on ligand binding

S1R residues	Drug tested[a]	Effect on binding	References
A13T/L28P/A86V	Haloperidol	Moderate	[71]
G87I	(+)-pentazocine	Large	[32]
G87 L	(+)-pentazocine	Large	[32]
G88I	(+)-pentazocine	Large	[32]
G88 L	(+)-pentazocine	Large	[32]
G91I	(+)-pentazocine	Large	[32]
G91 L	(+)-pentazocine	Large	[32]
C94	[^{125}I]IABM	Derivatized	[29]
C94A/V190C	(+)-pentazocine	No significant effect	[29]
H97A	(+)-pentazocine	Moderate	[32]
S99A	(+)-pentazocine, NE-100	Moderate	[31]
S101A	(+)-pentazocine	Large	[30]
Y103A	(+)-pentazocine	Large	[30]
Y103F	(+)-pentazocine, NE-100	Large	[31]
L105A/L106A	(+)-pentazocine, NE-100	Moderate	[31]
L105A/L106A/S99A	(+)-pentazocine	No significant effect	[31]
L105A/L106A/S99A	NE-100	Large	[31]
F107A	(+)-pentazocine	Large	[30]
R119A	(+)-pentazocine	Large	[30]
Y120A	(+)-pentazocine	Moderate	[30]
W121A	(+)-pentazocine	No significant effect	[30]
E123G	Haloperidol	No significant effect	[11]
S125A	(+)-pentazocine	Moderate	[30]
D126G	Haloperidol	Large	[11]
D126E	(+)-pentazocine	Moderate	[30]
T127A	(+)-pentazocine	Moderate	[30]
I128A	(+)-pentazocine	Large	[30]
E138G	Haloperidol	No significant effect	[11]
E144G	Haloperidol	No significant effect	[11]
V145A	(+)-pentazocine	No significant effect	[30]
F146A	(+)-pentazocine	Moderate	[30]
Y147A	(+)-pentazocine	No significant effect	[30]
E150G	Haloperidol	No significant effect	[11]
H154	4-NPPC12	Derivatized	[33]
H154A	4-NPPC12, (+)-pentazocine	No significant effect	[33]
E158G	Haloperidol	No significant effect	[11]
E163G	Haloperidol	No significant effect	[11]
M170C	[^{3}H]DTG	No significant effect	[33]
E172G	Haloperidol	Large	[11]
E172D	(+)-pentazocine	Large	[30]
Y173A	(+)-pentazocine	Large	[30]
Y173S[b]	Cholesterol	Large	[72]
R175A	(+)-pentazocine	Large	[30]

(continued)

Table 3.1 (continued)

S1R residues	Drug tested[a]	Effect on binding	References
T181A	(+)-pentazocine	No significant effect	[30]
F184A	(+)-pentazocine	No significant effect	[30]
D188G	Haloperidol	No significant effect	[11]
D188	[^{125}I]IACoc	Derivatized	[27]
T189A	(+)-pentazocine	No significant effect	[30]
F191A	(+)-pentazocine	No significant effect	[30]
D195G	Haloperidol	No significant effect	[11]
Y201S/Y206S[b]	Cholesterol	Large	[72]
E213G	(+)-pentazocine	No significant effect	[11]
D222G	Haloperidol	No significant effect	[11]
Deletion of 179–223	[^{125}I]IACoc	Large	[27]
Deletion of 189–223	[^{125}I]IACoc	Large	[27]
Deletion of 199–223	[^{125}I]IACoc	Large	[27]
Deletion of 209–223	[^{125}I]IACoc	Large	[27]
Deletion of 219–223	[^{125}I]IACoc	no significant effect	[27]
Deletion of 201–223	(+)-pentazocine	Large	[30]
Deletion of 209–223	(+)-pentazocine	Large	[30]
Deletion of 217–223	(+)-pentazocine	Moderate	[30]
Deletion of 119–149	Halperidol	Large	[71]

[a]Abbreviations: *[^{125}I]IABM* [methanesulfonothioicacid,S-((4-(4-amino-3-[^{125}I]iodobenzoyl)phenyl)methyl) ester, *4-NPPC12* N [3-(4-nitrophenyl)propyl] N dodecylamine, *[^{3}H]DTG* 1,3-di(2-tolyl)guanidine, *[^{125}I]IACoc* methyl-3-(4-azido-3-[^{125}I]iodo-benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate, *(+)-PPP* (+)-1-propyl-3-(3-hydroxyphenyl)piperidine

[b]In this study, the effects of substitutions on cholesterol binding were tested in the context of 20 amino acid synthetic peptides rather than the full-length receptor

Some substitutions are further from the binding site but have large effects on affinity. Most notably this includes R119A [30], which is in a loop connecting strands 4 and 5, with the sidechain pointing away from the binding pocket. Elevated crystallographic B-factors for this residue indicate that it may be relatively mobile. However, the basic sidechain of R119 is at the oligomeric interface and along with His116 makes an intermolecular salt bridge to D195 of an adjacent subunit. R119 also makes an intermolecular hydrogen bond to T198. Thus, the R119A substitution may destabilize oligomerization, which has been correlated with ligand binding [32]. In that study, substitutions at G87 and G88 within SBDL1 also resulted in decreased ligand binding and decreased oligomer stability. G87 and G88 can now be seen to form a Type I′ turn in the β-hairpin turn of the SBDL1. Position 88 is then required to be a glycine for the stability of the turn and is strictly conserved (Fig. 3.1). However, the β-hairpin turn is found also at the 3-fold symmetry axis of the trimer and thus disruption of the turn is likely to cause also disassembly of the oligomer. Position 87 could, in theory, tolerate non-glycine amino acids, but the larger sidechain would likely clash with the adjacent subunit.

C-terminal deletions on ligand binding can also be understood in terms of the structure. Deleting five or seven C-terminal residues, which has small or modest effects on ligand binding, removes approximately a single turn of helix E that has no long-range contacts. By contrast, deleting fourteen C-terminal residues would be expected to remove several long-range contacts that helix E makes with SBDL1 and the loop between helices B and C. Indeed, this deletion results in little or no ligand binding [27, 30].

Additional amino acids have been suggested to be in direct contact with ligand because they are derivatised by photoreactive probes based on S1R ligands. This includes C94 [29], H154 [33], and D188 [27] (Fig. 3.3b). H154 is in contact with ligand at the narrow end of the cupin barrel, whereas C94 is at the other end of the barrel in strand 2. Although C94 is in a strand within SBDL1, the reactive thiol sidechain points away from the binding pocket, suggesting that either flexibility in the strand or mobility of the ligand within the binding site allows derivatization, or that the sidechain lies along the binding pathway. Crystallographic B-factors are increased for both complexes in ligand atoms that are nearer the 'open' end of the cupin barrel suggesting that bound ligands may have increased degrees of freedom here. D188 is at the end of helix D and within SBDL2, but like C94, it is at the more open end of the cupin barrel and while there are no direct interactions with bound ligand, it can be envisioned how flexibility in the protein or ligand might facilitate derivatisation.

S1R binds a chemically diverse range of small molecules, which has motivated development of S1R ligand pharmacophore models that can now be understood in the context of the receptor structure (Fig. 3.4). The pharmacophore models have been developed from analyses of known S1R ligands as well as binding affinity measurements of systematically substituted panels of ligands [34–50]. The first pharmacophore models developed after separation of the S1R binding site from the Sigma 2 Receptor binding site [51, 52] were based on disubstituted piperidines [36] and N-substituted phenylalkylamines [37]. Subsequent models have been largely consistent with these models in that a central basic amine nitrogen atom, or more generally a positive ionizable feature, is flanked on either side, in a more or less linear arrangement by a set of hydrophobic features (Fig. 3.4a). On one side of the nitrogen is a primary hydrophobic feature centered 6–10 Å from the nitrogen atom, and on the other side is a secondary hydrophobic feature that is centered 2.5–3.9 Å from the nitrogen atom, with the latter being tolerant of bulky substituents with little change in affinity [37].

PD144418 or 4-IBP fit well to the S1R pharmacophore, and as noted above the key amino acid stabilizing the positive ionizable feature is E172, with D126 and Y103 playing a supporting role (Fig. 3.4b). Although some degree of ambiguity in ligand orientation can arise in fitting of the ligands to the experimentally derived electron density, the binding site for the larger, primary hydrophobic feature appears to be at the membrane proximal and more open end of the cupin barrel, while the secondary hydrophobic feature is within the narrower end of the cupin β-barrel that is further from the membrane. Further support for this orientation comes from the photoreactive probes of the binding pocket: C94 and D188 at the open end of the cupin barrel react with the photoprobes [^{125}I]IABM [29] and [^{125}I]IACoc [27], respectively, which have reactive groups on the primary hydrophobic feature, whereas H154 at the smaller end of the cupin barrel reacts with the photoprobe 4-NPPC12 [33], which has a reactive nitrophenol on the secondary hydrophobic feature.

Several research groups have expanded on the earlier S1R pharmacophore model, with some variation between models expected depending on the panel of ligands used to develop the models. Laggner et al., for example, split the primary hydrophobic group into two hydrophobic groups and inserted a third hydrophobic group between it and the central nitrogen [43] (Fig. 3.4c). In the model developed by Zampieri et al., the primary hydrophobic group is divided into a hydrophobic aromatic closer to the central positive ionizable feature and another hydrophobic site further out [46] (Fig. 3.4d). The Zampieri model includes also a hydrogen bond acceptor site, and from the S1R structures the sidechains of residues Y103 and T181 are close enough to be potential hydrogen bond donors. Substitutions at Y103 are known to affect drug binding [30, 31], however Y103 interacts with the critical residue E172 and also ring stacks with the ligand at the membrane-proximal end of the binding pocket. A T181A substitution does not affect (+)-pentazocine binding [30], however that ligand does not appear to have the expected hydrogen bond acceptor.

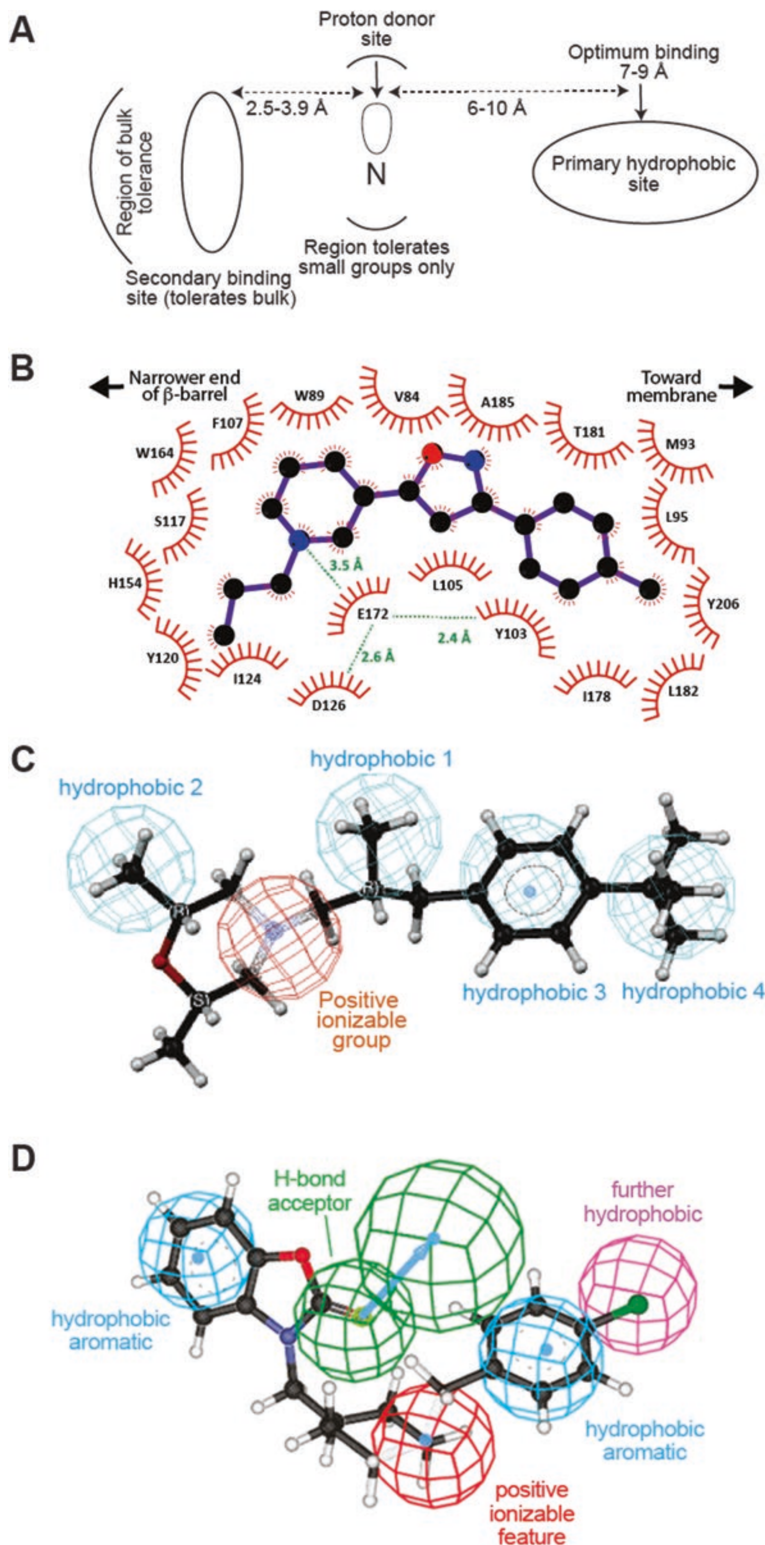

Fig. 3.4 S1R ligand binding site and pharmacophore models. (**a**) Pharmacophore model derived from N-substituted phenylalkylamines [36, 37]. Schematic based on [37]. (**b**) A schematic diagram of the S1R residues lining the binding pocket for PD144418 based on PDB file 5HK1. The indicated distances are from Cδ of E172 to N4 of PD144418, from an Oδ of D126 to an Oε of E172, and from OH of Y103 to an Oε of E172. The leftmost residue, H154, is in β-strand 8 at the narrower end of the β-barred-like fold, whereas the rightmost residue, Y206, is in the membrane-proximal helix E. The figure is an adaptation of a LigPlot output [75]. (**c**) Pharmacophore model of Laggner et al. mapped onto fenpropimorph [43]. (**d**) Pharmacophore model of Zampieri et al. mapped onto a benzooxazolone derivative [46]

3.4 Structural Implications for Membrane Topology

Sequence-based transmembrane helix predictors had reliably predicted for S1R an N-terminal transmembrane helix at residues ~9–30, which was confirmed by the structure. The transmembrane helix may also contain a signal peptide directing it to the ER membrane [53], and homologs of S1R and the fungal sterol isomerases can be found in γ- and δ-proteobacteria that are 35–38 % identical in sequence (Fig. 3.1) but lack the first transmembrane helix. A second transmembrane helix was expected based on analysis of amino acid hydrophobicity [10] and predictors of transmembrane helices [54]. Studies of the S1R membrane topology indicated that the N- and C-termini of S1R reside on the same side of the membrane, consistent with an even number of transmembrane domains [2, 10, 55]. In addition, solution NMR studies of a truncated form of S1R reconstituted into a mixture of detergent and lipids were consistent with a helical conformation of the SBDL1 and assigned to a second transmembrane domain [54]. The structure of the intact receptor however, indicates only a single transmembrane helix at the N-terminus, and that the SBDL1 adopts a β-hairpin conformation [19].

Notably, membrane topology studies of S1R have been ambiguous: in oocyte studies both termini were determined to be in the cytosol [10], whereas in CHO cells [2] or human embryonal kidney cells [55] both termini were found to be in the ER lumen or on the extracellular side of the plasma membrane, respectively. The surprising single transmembrane domain architecture of S1R places the vast majority of the protein on one side of the membrane and leaves only a handful of poorly conserved residues on the other side. Thus, the S1R membrane topology makes it difficult to rationalize how the receptor could interact with proteins both in the cytoplasm and in the ER lumen. Interactions with several proteins or regions of protein that are found exclusively in the cytoplasm or the nucleus, including the C-terminus of the GluR1 subunit of the ionotropic glutamate receptor [57], STIM1 [58], and emerin [12], are consistent with S1R being a Type I membrane protein with a cytosolic C-terminus. However, an interaction at the C-terminus of S1R with BiP has been shown in cell extracts [2] and *in vitro* [25]. Although BiP can relocalize to the cytoplasm at low concentrations [56], it is predominantly found in the ER lumen. Thus, further work is necessary to understand the biological relevance of this interaction.

3.5 Oligomerization

Early evidence for the formation of S1R oligomers came from studies in which selective photoreactive-probes reacted with higher molecular weight species [28]. More recently, it has been shown that receptor expressed and purified from either *E. coli* [32] or insect cells [19] forms a mixture of oligomeric forms and that ligands stabilize the oligomeric forms of the protein [32]. The existence of oligomeric forms of S1R in membranes has been shown also in COS-7 cells by FRET after co-transfection with S1R-GFP2 and S1R-YFP [59], and, consistent with the *in vitro* studies [32], the introduction of ligands (specifically antagonists) stimulated the formation of higher order species.

The structures of ligand-bound S1R observed by X-ray crystallography are homotrimeric, which was surprising in light of previous studies suggesting that an even-number of subunits was likely. For example, only ~50 % of S1R preparations were observed to bind (+)-pentazocine [60], and the photoreactive-probe 4-NCCP12 derivatizes no more than 50 % of the receptor molecules [33]. The proteins in these binding studies were recombinantly expressed and purified from *E. coli*, and it remains possible that on average about half of the proteins are fully folded under those conditions. However, a similar level of derivatization by 4-NCCP12 was seen for S1R expressed in COS-7 cells and guinea pig liver microsomes. In addition, analytical size exclusion chromatography results from Gromek et al. [32], were most consistent with a tetramer and a larger species consisting of approximately 6–8 subunits, whereas cellular fluorescence data was

consistent with mostly monomeric and dimeric forms of the receptor [59]. The role of S1R oligomerization, including that of the alternative oligomeric states, remains unknown.

In the S1R trimer structure determined by X-ray crystallography, extensive polar and nonpolar inter-subunit contacts are made over a large surface area [19]. Some contacts, including a hydrophobic cluster formed by the three phenylalanines at position 191, are found along the 3-fold symmetry axis. Other notable contacts are made toward the periphery of the trimer in which residues from the loop connecting strands 6 and 7 interact with residues in an adjacent subunit. W136, at the end of strand 6, is at the center of a hydrophobic core of residues from an adjacent subunit. Packing on top and at the side of W136 are F83, M90, and A92, and making contacts below the tryptophan are T109, A110, and L111. These residues are from strands 1 and 2, and the loop between strands 3 and 4, respectively, of the adjacent subunit. Intriguingly, W136 and T141 are within the stretch of residues ~135–165 that are predicted from amino acid sequence to have a high degree of intrinsic disorder. This suggests that despite a large number of inter-subunit contacts, the stabilizing effects of these enthalpic contacts may be offset by entropic costs from restricting flexibility in these residues.

Other inter-subunit contacts include polar interactions between the sidechains of T141 of strand 6 and the sidechains of H54 and E55 in helix B, and a bifurcated hydrogen bond between the sidechain of Q194 and the peptide bond joining A183 and F184. Many of the sidechains forming inter-subunit contacts, including those of H54, E55, W136, T141, F191 and Q194, are not highly conserved in the fungal sterol isomerases suggesting that this observed mode of oligomerization may be unique to the S1Rs.

3.6 Future Directions

The ligand-bound structures of S1R [19] have provided an enormous leap forward in the study of S1R and have helped to unify and extend several decades of biochemical studies. Yet, numerous structural questions remain. In this section, we highlight some of the most interesting unanswered questions in light of the receptor structure.

What Are the Conformational Changes Associated with Receptor Activation? The receptor structures are bound to two different S1R ligands, one of which is a known antagonist and the other may be an agonist. Nonetheless, the two protein structures are very similar (0.4 Å all atom RMSD) [19]. Thus, the mechanism of receptor activation remains a mystery. Also unknown is whether the receptor adopts multiple conformations with different activities or only exists in an 'on' or an 'off' state. Ultimately the structures of S1R in the apo state and the agonist bound state will be needed to provide insights into the conformational changes associated with receptor activity. Further studies of changes in local and global flexibility in the receptor associated with ligand binding is likely to be necessary to fully understand receptor activation [61].

Where Is the Interaction Site for Protein-Protein Interactions? Identification of the structural elements and amino acids involved in the large number of reported protein-protein interactions (reviewed in [1]) is required to understand the specificity of S1R signaling. It is possible that the interface for protein-protein interactions is only accessible in the monomeric form [32, 62, 63], however, the surface-exposed helices A and B are attractive potential sites of interaction based on the trimeric structures. Residues 61–65 in helix B form a putative SUMO interaction site, and peptide interference assays are consistent with the binding of the GluR1 cytoplasmic tail near this region [57]. Furthermore, the helices lie against the large β-sheet enclosing the ligand binding site, and helix B contacts L106, which has been proposed to be involved in discriminating between agonist and antagonist [31]. Thus, one can begin to hypothesize about potential allosteric pathways between the ligand and protein binding sites. The prospects for investigating such hypotheses are good: several protein-protein interactions of S1R have been directly observed

[55, 62–65], and advances in recombinant expression and purification of the receptor [19, 54, 60, 66] make it likely that biophysical assays will soon complement *in vivo* studies of protein-protein interactions. Such studies may also help to reconcile contradictory information on the S1R membrane topology.

How Is the S1R Ligand Binding Site Able to Accommodate Chemically Diverse Ligands? Despite being bound to different ligands, the S1R ligand binding site architecture is essentially unchanged in the two crystallographic structures. Thus, to understand the physical properties of the S1R binding pocket additional structures of S1R bound to other ligands are needed, and likely also information on dynamics of both the protein and the bound drug. Especially informative may be structural information on receptor complexes with neurosteroids and single-chain lipid-like compounds, since they lack positive ionizable features. With regard to the binding of diverse ligands, the β-sheet structure of the S1R binding pocket may be expected to provide the flexibility needed for adaptation of the binding site [67]. A useful comparator is the fatty-acid binding protein family, since they are able to bind both long-chain fatty acids and bile salts, and have also a central binding pocket buried between antiparallel β-sheets [68]. β-sheets also facilitate large-scale correlated motions [69], which may be important for allosteric signaling outward from the buried ligand binding pocket.

Does Oligomerisation Regulate S1R Interactions? Based on previous work the trimeric structure of S1R was unexpected. Available data suggest that this is not a function of the protein expression system since protein prepared similarly to that which was crystallized also displays polydisperse oligomerisation [19]. Thus, the consequences of oligomerisation for receptor function, including downstream protein-protein interactions, need to be better understood.

How Does Drug Get In and Out of the Binding Pocket? The central fold of S1R is β-barrel-like, having a narrower, essentially closed, end where the two sheets are closely apposed, and a more open end in which the longer strands of the large sheet curve toward the smaller sheet. However the open end of the central β-barrel is capped by helices D and E and there is no obvious entry or exit pathway to the ligand binding site. Being related to the sterol isomerases would suggest access occurs through the region closest to the membrane, which would be also consistent with the presumed binding pathway for enzymes of the cupin fold family. However, the β-strands at the narrow end of the barrel have elevated crystallographic B-factors and are predicted from sequence to be flexible, thus dynamics here may provide a transient pathway from solvent to the ligand binding site.

What Is the Molecular Basis of S1R Chaperone Activity? No further studies on the chaperone activity have appeared since Hayashi and Su first identified such activity and linked it to the C-terminal half of S1R [2]. Important questions remain, such as whether the full-length receptor exhibits similar activity and whether the chaperone activity is ligand-dependent. Also important is to test the relatedness of the S1R chaperone domain function to known families of protein chaperones [70].

Many of the questions outlined above have been elucidated previously, but the new S1R structures provide a firmer foundation from which these questions can be approached. Without a doubt S1R will continue to throw up surprises as the answers to these questions become more clear.

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4 Fluorinated PET Tracers for Molecular Imaging of σ_1 Receptors in the Central Nervous System

Frauke Weber, Peter Brust, Erik Laurini, Sabrina Pricl, and Bernhard Wünsch

Abstract

At first the role of σ_1 receptors in various neurological, psychiatric and neurodegenerative disorders is discussed. In the second part, the principle of positron emission tomography (PET) is described and the known fluorinated PET tracers for labeling of σ_1 receptors are presented. The third part focuses on fluoroalkyl substituted spirocyclic PET tracers, which represent the most promising class of fluorinated PET tracers reported so far. The homologous fluoroalkyl derivatives **12**–**15** show high σ_1 affinity (K_i = 0.59–1.4 nM) and high selectivity over the σ_2 subtype (408–1331-fold). The enantiomers of the fluoroethyl derivative fluspidine **13** were prepared and pharmacologically characterized. Whereas the (*S*)-configured enantiomer (*S*)-**13** (K_i = 2.3 nM) is 4-fold less active than the (*R*)-enantiomer (*R*)-**13** (K_i = 0.57 nM), (*S*)-**13** is metabolically more stable. The interactions of (*S*)-**13** and (*R*)-**13** with the σ_1 receptor were analyzed at the molecular level using the 3D homology model. In an automated radiosynthesis [^{18}F](*S*)-**13** and [^{18}F](*R*)-**13** were prepared by nucleophilic substitution of the tosylates (*S*)-**17** and (*R*)-**17** with K[^{18}F]F in high radiochemical yield,

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high radiochemical purity and short reaction time. Application of both enantiomers $[^{18}F](S)$-**13** and $[^{18}F](R)$-**13** to mice and piglets led to fast uptake into the brain, but $[^{18}F](R)$-**13** did not show washout from the brain indicating a quasi-irreversible binding. Both radiotracers $[^{18}F](S)$-**13** and $[^{18}F](R)$-**13** were able to label regions in the mouse and piglet brain with high σ_1 receptor density. The specific binding of the enantiomeric tracers $[^{18}F](S)$-**13** and $[^{18}F](R)$-**13** could be replaced by the selective σ_1 ligand SA4503.

Keywords

Neuroimaging • PET, synthesis • Radiosynthesis • σ_1 receptors • Ligand receptor interaction • Enantioselective kinetics • Non-covalent quasi irreversible binding

4.1 Introduction: σ_1 Receptors in Brain Diseases

σ_1 receptors play a major role in various pathological conditions in the periphery (e.g. vascular diseases, cancer) and in the central nervous system (CNS), where they are involved in various neurological, psychiatric and neurodegenerative disorders. Non-invasive imaging of σ_1 receptors by positron emission tomography (PET) can be useful for studying the pathophysiology of these CNS diseases. Moreover, a PET tracer for labeling of σ_1 receptors can be used for target validation, visualization and quantification of metabolic and biochemical processes as well as diagnosis and prognosis of a particular disease [1, 2].

4.1.1 Pain

It has been shown that the σ receptor system functions as endogenous anti-opioid system. Whereas σ_1 agonists, such as (+)-pentazocine (Fig. 4.1) lead to reduced opioid receptor-mediated analgesia, the opposite effect is produced by σ_1 antagonists, e.g. haloperidol [3]. The combination of opioid analgesics with σ_1 antagonists allows the reduction of opioid dose, while maintaining strong analgesia but reducing opioid- mediated side effects. Furthermore, opioid receptor mediated analgesia is potentiated by downregulation of σ_1 receptors [4]. In addition to the modulation of opioid mediated analgesia, σ_1 receptor antagonists show promising analgesic activity in various neurogenic pain models. The role of σ_1 receptors in neuropathic pain conditions was confirmed by σ_1 receptor knock-out mice: capsaicin could not induce mechanical allodynia in σ_1 receptor knock-out mice, but it was able to induce mechanical allodynia in wild-type mice. This mechanical allodynia was inhibited dose-dependently by σ_1 antagonists. Moreover, selective σ_1 agonists were able to reverse this analgesic effect [5]. The most developed drug in this field is the σ_1 antagonist S1RA (Fig. 4.1), which showed high analgesic activity in various models of neurogenic pain. After successful completion of the phase I clinical trial, a phase II clinical trial with S1RA for the treatment of neuropathic pain caused by various conditions is currently ongoing. Moreover, S1RA is investigated as add-on therapy to analgesic opioids with the aim to enhance the analgesic effect and reduce dose and adverse side effects pf the opioid [6, 7].

4.1.2 Depression

There is strong evidence that σ_1 receptors are involved in the pathophysiology of depression. It was observed that σ_1 receptor knock-out mice develop a depressive-like behavior. [8] In animal models of depression (e.g. forced swim

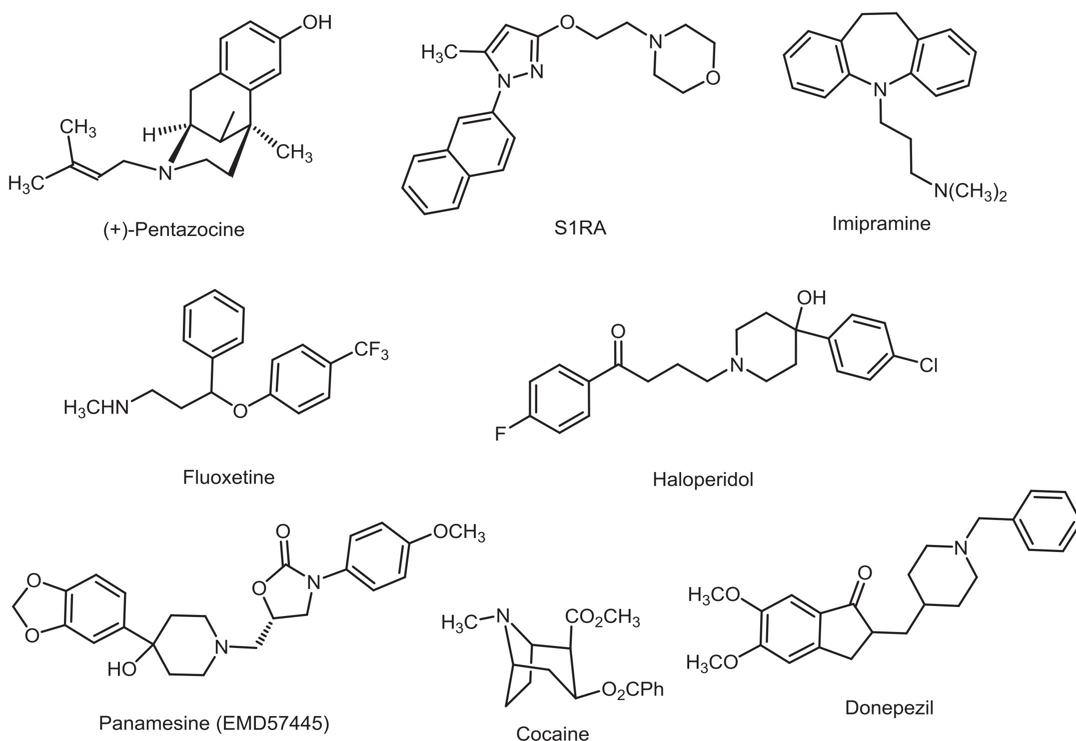


Fig. 4.1 Important σ_1 receptor ligands: (+)-pentazocine, the prototypical benzomorphan σ_1 receptor agonist; S1RA in phase II clinical trials for pain management /neuropathic pain, add-on to opioids); antidepressants imipramine and fluoxetine; antipsychotics haloperidol and panamesine; cocaine as example for an abuse compound; anti-Alzheimer drug (acetylcholinesterase inhibitor) donepezil

test) some σ_1 agonists showed antidepressive properties [9, 10]. Antidepressant activity of σ_1 agonists was also observed in animal models of Alzheimer's disease (AD) [11, 12]. In addition to their main pharmacological mechanism several clinically used antidepressants reveal high to moderate σ_1 affinity (Ki(σ_1) = 20–200 nM). In Fig. 4.1 the tricyclic antidepressant imipramine and the selective serotonin reuptake inhibitor fluoxetine are shown exemplarily. It is assumed that the interaction of these drugs with the σ_1 receptor contributes to their overall antidepressive effects. Moreover, a downregulation of σ_1 receptors in the striatum, hippocampus and cerebral cortex was reported after repeated treatment of rats with imipramine and fluoxetine [13]. The same effect was observed after repeated treatment with the σ_1 agonist (±)-pentazocine [14].

4.1.3 Psychosis

In addition to its dopamine D_2 receptor antagonistic activity the clinically used prototypical antipsychotic haloperidol (Fig. 4.1) reacts as a potent antagonist at σ_1 receptors ($K_i(\sigma_1)$ = 3.9 nM). Moderate to high σ_1 affinity has also been reported for other clinically used antipsychotics. It is supposed that σ_1 antagonistic activity contributes significantly to the observed antipsychotic activity of these antipsychotics. Recently, a correlation between a polymorphism within the σ_1 receptor gene and increased risk of schizophrenia was reported [15]. During the past 10 years five σ_1 antagonists (panamesine (EMD57445), eliprodil (SL82.0715), rimcazole (BW234U), BMY14802 (BMS181100), DuP734) entered clinical trials for the treatment of schizophrenia [16]. In Fig. 4.1 panamesine is depicted exemplarily for this class of ligands.

4.1.4 Addiction

Activation of σ_1 receptors contributes considerably to plasticity processes underlying reinforcement and addiction. The high density of σ_1 receptors in addicted rats may play a crucial role in the reinforcing effects of addictive drugs, such as methamphetamine, cocaine and ethanol [17]. After chronic self-administration of methamphetamine to rats, σ_1 receptor upregulation has been found [18–20]. It was postulated that methamphetamine-induced dopamine D_2 autoreceptor downregulation leads to increased protein kinase A activity resulting in increased production of σ_1 receptors [21]. The existence of heteromeric receptors consisting of both σ_1 and dopamine D_1 receptors supports the hypothesis of the involvement of σ_1 receptors in addictive processes [22]. The behavioral effects caused by methamphetamine could be inhibited by the σ_1 antagonist MS-377. Cocaine (Fig. 4.1) binds with high affinity at the σ_1 receptor and behaves as σ_1 receptor agonist [23]. Cocaine responses of addicted rats could be blocked by σ_1 antagonists. Whereas the σ_1 antagonist BD1047 diminishes ethanol induced behavioral effects, the σ_1 agonist PRE-84 reinforced these addictive responses [24].

4.1.5 Alzheimer's Disease

Although σ_1 receptors are not involved in learning and memory processes, since these processes cannot be modulated by σ_1 agonists or σ_1 antagonists, they are involved in diseases associated with memory deficit. The role of σ_1 receptors was investigated in a mouse model of Alzheimer's disease, which was generated by central application of amyloid β_{25-35}. It was shown that the selective σ_1 agonist (+)-pentazocine could attenuate dose-dependently the memory deficits occurring seven days after amyloid β_{25-35} injection. Acetylcholinesterase inhibitors, which are clinically used for the treatment of Alzheimer's disease, represent the first-line therapy. It has been reported that donepezil (Fig. 4.1) not only inhibits the acetylcholinesterase but also activates σ_1 receptors. This interaction with σ_1 receptors was postulated to contribute to the overall neuroprotective and anti-amnesic effects of donepezil [25]. In a PET study with early Alzheimer's patients a low density of σ_1 receptors was observed. It may be concluded that activation of σ_1 receptors might be a useful strategy for the treatment of Alzheimer's disease.

4.2 Fluorinated PET Tracers for σ_1 Receptors

4.2.1 Principle of Positron Emission Tomography (PET)

Positron emission tomography (PET) represents a promising modality for studying biological processes in a non-invasive manner. For PET a positron emitter is required, i.e. a radioactive nuclide with increased number of protons in the nucleus. This type of isotope can release a positron and a neutrino (ν) upon conversion of a proton into a neutron. The emitted positron travels in matter until it meets its antiparticle, an electron. The positron and the electron react with each other in an annihilation process, i.e. transformation of the complete mass of the particles into irradiation energy. The annihilation process generates two gamma quants with an energy of 511 keV, which move in opposite directions (angle = 180°). A signal is only accepted as an annihilation event, when two gamma quants are registered simultaneously in opposite directions. The registration of two signals allows the identification of the origin of the irradiation and thus the original position of the PET tracer (Fig. 4.2).

The most commonly used non-metallic positron emitters are ^{11}C, ^{13}N, ^{15}O, and ^{18}F. The corresponding decay reactions and half-lives are depicted in Table 4.1. Since the radionuclides ^{11}C, ^{13}N and ^{15}O decay with very short half-lives, the isotope ^{18}F with a half-life of 110 min represents the most interesting radionuclide for the development of PET tracers. In particular labeling with ^{18}F does not require a cyclotron on bedside. However, C, O, and N atoms are present in almost all pharmacologically active compounds.

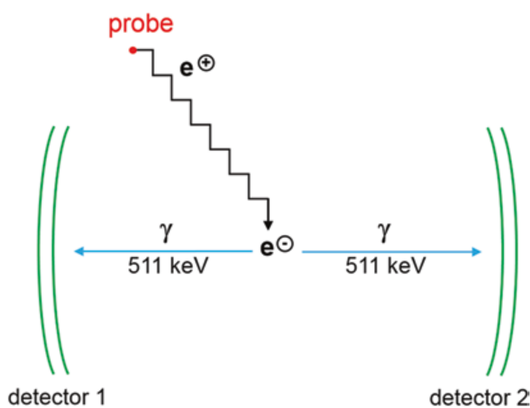


Fig. 4.2 Principle of PET

The corresponding radionuclides can be introduced without changing the structure of the compound. In contrast, F atoms are not present in all drugs, and therefore a potent fluorine containing drug has to be developed first before the corresponding ^{18}F–labeled]PET tracer can be developed. This report is focusing on fluorinated PET tracers for imaging of σ_1 receptors.

4.2.2 Fluorinated PET Tracers for Imaging of σ_1 Receptors

In the literature a great variety of fluorinated PET tracers for imaging of σ_1 receptors is reported [26]. In the following part the fluorinated PET tracers are classified into compounds bearing ^{18}F at the aromatic ring (Fig. 4.3) and compounds with an aliphatic ^{18}F atom (Fig. 4.4).

Although methods for the introduction of [^{18}F] fluoride into the aromatic ring have been reported, very often several further reaction steps are required to obtain the final PET tracer. The additional reaction steps lead to a longer production time and thus reduced radiochemical yields.

Cyclopropyl-(4-nitrophenyl) methanone served as starting material for the synthesis of [^{18}F]haloperidol and [^{18}F]BMY14802. (Fig. 4.3) At first [^{18}F]fluoride was introduced by nucleophilic aromatic substitution of the nitro moiety with Cs[^{18}F]F. Subsequent cleavage of the cyclopropyl ring with HCl and nucleophilic substitution of the resulting chlorobutyrophenone with

Table 4.1 Decay reactions and half-lives of non-metallic positron emittors

Decay reaction		Half-life
$^{11}_{6}C \rightarrow$	$^{11}_{5}B + e^{+}$ (1.0 MeV) + ν	20.4 min
$^{13}_{7}N \rightarrow$	$^{13}_{6}C + e^{+}$(1.2 MeV) + ν	9.96 min
$^{15}_{8}O \rightarrow$	$^{15}_{7}N + e^{+}$(1.7 MeV) + ν	2.03 min
$^{18}_{9}F \rightarrow$	$^{18}_{8}O + e^{+}$(0.6 MeV) + ν	109.8 min

the corresponding N-heterocycle provided the PET tracers [^{18}F]haloperidol and [^{18}F]BMY14802 [27–29], whereby the synthesis of [^{18}F] BMY14802 required additional reduction of the ketone [30]. The selectivity of haloperidol and BMY-14802 for the σ_1 receptor is rather low, since both compounds show strong interactions with dopamine and σ_2 receptors as well. Therefore, both PET tracers can be used for the determination of uptake, distribution, penetration of the blood brain barrier, metabolism, and further pharmacokinetic parameters. The selective labeling of σ_1 or dopamine receptors with these tracers is however not possible.

The [^{18}F]fluorobenzylamines [^{18}F]**1**, [^{18}F]**2a**, and [^{18}F]**2b** were obtained in a four-step process. Nucleophilic substitution of 2- or 4-nitrobenzaldehyde with Cs[^{18}F]F or K[^{18}F]F led to the radioactively labeled fluorinated benzaldehyde. Reduction of the aldehyde and nucleophilic substitution afforded the fluorinated benzyl iodide, which was coupled with the corresponding piperidine to provide the PET tracers [^{18}F]**1**, [^{18}F]**2a**, and [^{18}F]**2b** in 3–10 % radiochemical yield. In rats and monkeys the PET tracer [^{18}F]**1** showed rapid brain uptake and fast washout. Replacement studies with haloperidol confirmed selective labeling of σ_1 receptors without addressing the σ_2 subtype [31]. In rat distribution experiments high penetration of

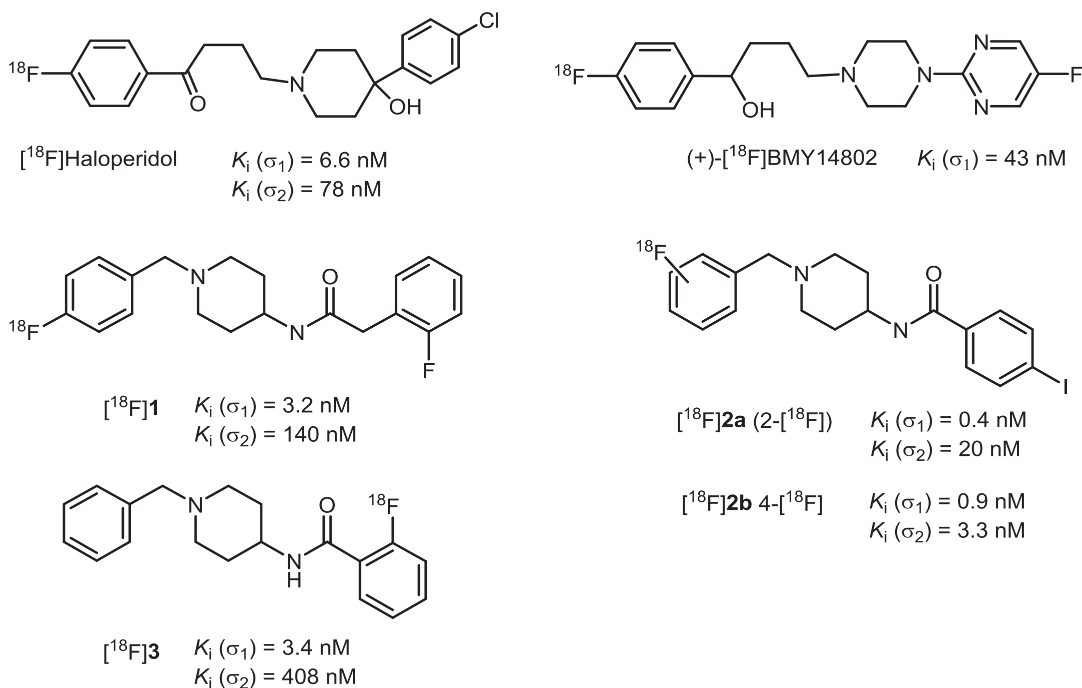


Fig. 4.3 Structure, σ_1 and σ_2 affinity of PET tracers with aromatic ^{18}F

[^{18}F]**2a** into the brain was detected. However, the high liver uptake could not be blocked by haloperidol. Furthermore, the high σ_2 affinity of [^{18}F]**2a** and [^{18}F]**2b** has to be considered [32].

In contrast to the radiosynthesis of the previous compounds, the radiosynthesis of [^{18}F]**3** was performed in a single step at the end of the synthesis by nucleophilic aromatic substitution of the corresponding 2-NO_2 derivative with K[^{18}F]F/Kryptofix 2.2.2/K_2CO_3 complex. The radiochemical yield was 5–10 % (decay corrected). In mice, high uptake of the PET tracer [^{18}F]**3** in the brain and peripheral organs was observed. The specific binding could be displaced by administration of haloperidol [33].

Fluorinated PET tracers with [^{18}F]fluoride bound at a sp^3-hybridized C-atom (aliphatic C-atom) are prepared by nucleophilic substitution (S_N2 reaction) of mesylate ([^{18}F]**4**, [^{18}F]**5**) or tosylate precursors ([^{18}F]**8**, [^{18}F]**9**, [^{18}F]**10**, [^{18}F]fluspidine). This transformation is usually performed as the last step of the synthesis, i.e. the labeled compound is produced and purified without further transformations.

In an alternative strategy, precursor molecules with a phenol or other nucleophilic functional group are coupled with [^{18}F]fluoroethyl ([^{18}F]FE-SA4503, [^{18}F]**6**) or [^{18}F]fluoromethyl ([^{18}F]FM-SA4503, [^{18}F]**7**) moieties at the end of the synthesis. In these cases the fluorinated reagents [^{18}F]FCH_2CH_2OTos and [^{18}F]H_2CBrF have to be prepared first by nucleophilic substitution of $TosOCH_2CH_2OTos$ and H_2CBr_2 with K[^{18}F]F/Kryptofix system, respectively. Whereas the production of [^{18}F]FCH_2CH_2OTos represents a standard procedure in radiochemistry [34], the synthesis of the [^{18}F]H_2CBrF is more challenging and not available everywhere.

In a first human study, the fluoropropyl derivative [^{18}F]**4** did not reach a transient equilibrium in the brain within 3 h after injection of the radiotracer. A significant washout of the radiotracer from the brain was not observed. Moreover, instability of the radiotracer [^{18}F]**4** was reported [35, 36]. In rodents, the fluoroethyl derivative [^{18}F]**5** showed a much faster clearance from the brain than [^{18}F]**4**. Human studies with [^{18}F]**5** have not been reported thus far [37–39].

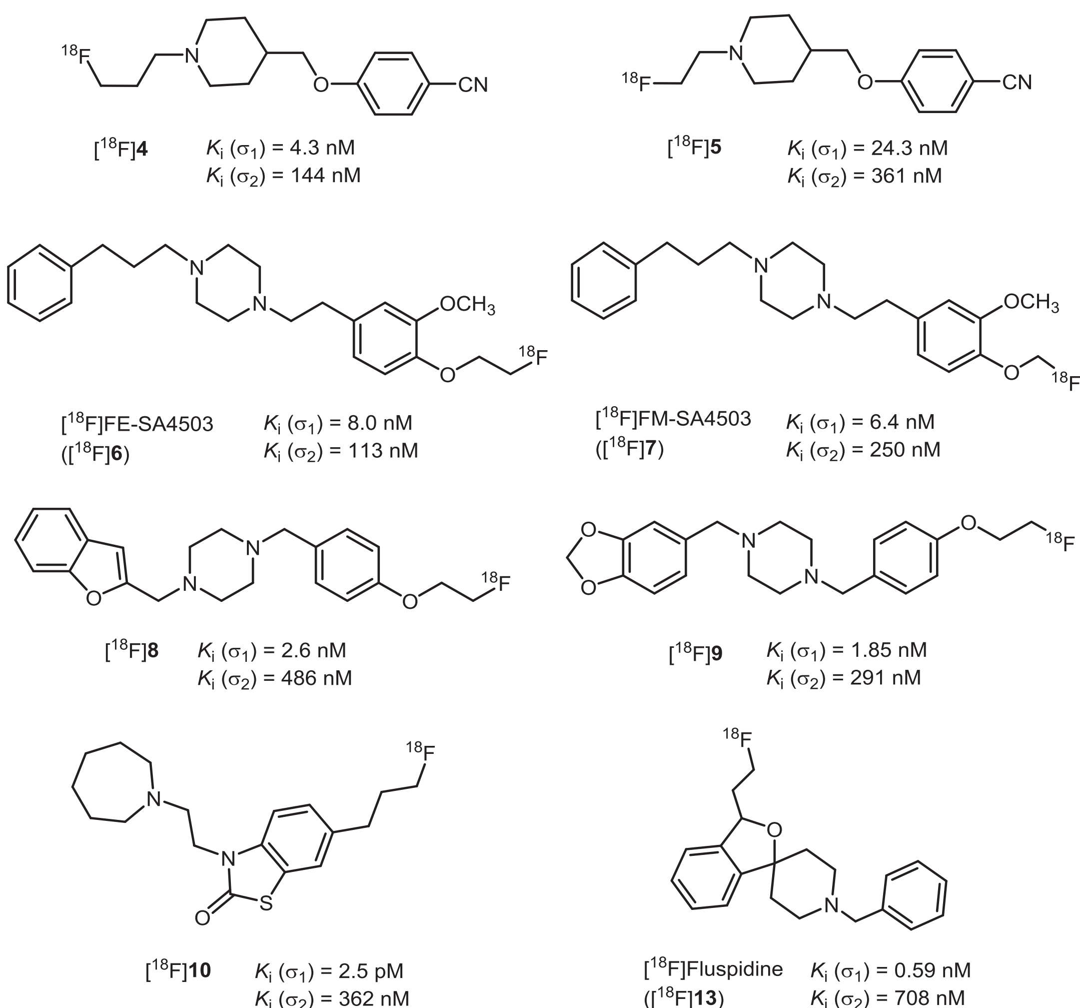


Fig. 4.4 Structure, σ_1 and σ_2 affinity of PET tracers with aliphatic ^{18}F

Originally a low σ_1: σ_2 selectivity of FE-SA4503 (**6**) was reported [40, 41], which was later corrected to be in the range of 14 [42]. Therefore the PET tracer [^{18}F]FE-SA4503 ([^{18}F]**6**) was investigated in various animal models. A PET study with rhesus monkeys resulted in a fast uptake of [^{18}F]**6** in the brain and enrichment of [^{18}F]**6** in σ_1 receptor rich regions, but the receptor ligand binding did not reach an equilibrium within 90 min [40]. The fluoromethoxy derivative [^{18}F]FM-SA4503 ([^{18}F]**7**) represents an uncommon PET tracer, since fluoromethoxy derivatives usually undergo fast metabolic degradation and extensive defluorination due to the acetalic nature of this group. However, these reactions were not observed for [^{18}F]**7**. Moreover, replacement studies with haloperidol in monkeys revealed a higher specific binding for [^{18}F]**7** than for [^{11}C]SA4503 rendering [^{18}F]**7** a more potent PET tracer for labeling of σ_1 receptors in the brain [41].

The synthesis and biological evaluation of the [^{18}F]fluoroethoxy and [^{18}F]fluoroalkyl labeled PET tracers [^{18}F]**8**, [^{18}F]**9**, [^{18}F]**10**, and [^{18}F]fluspidine ([^{18}F]**13**) were reported very recently. In animal studies with monkeys and mice, the biodistribution of the PET tracers was analyzed. They show accumulation in regions of the CNS and the periphery with high σ_1 receptor density. The specificity of σ_1 receptor binding was proven

by displacement experiments with haloperidol [43–45]. In σ_1 receptor knock-out mice the extraordinarily potent tracer [^{18}F]**10** (K_i = 2.5 pM) [44] showed rapid brain uptake and rapid clearance without specific interaction with any other brain target. In animal studies signs of toxicity could not be detected. Due to its high σ_1 receptor affinity and high specificity, [^{18}F]**10** is currently evaluated for imaging of σ_1 receptors in various neurological disorders, such as chronic pain and Alzheimer's disease [46].

The spirocyclic PET tracer [^{18}F]fluspidine ([^{18}F]**13**) [47], which is the only fluorinated PET tracer with a center of chirality, belongs to the most promising PET tracers reported so far and will be discussed in more detail in part 3 "Spirocyclic PET tracers" of this report.

4.3 Spirocyclic PET Tracers

4.3.1 Homologous Fluoroalkyl Derivatives 12–15

The development of spirocyclic PET tracers started with the 2-benzofuran **11** [48, 49]. It was found that **11** interacts with very high affinity with σ_1 receptors (K_i = 1.1 nM). Since the σ_2 affinity (K_i = 1280 nM) is very low, **11** shows an excellent 1100-fold selectivity for σ_1 receptors over the σ_2 subtype. Cross reaction with other targets could not be detected during a screening against more than 60 other receptors, ion channels, transporters and enzymes. **11** did not interact with the hERG channel [50]. The hERG channel is a voltage gated K^+-channel in the heart, whose blockade can lead to life threatening arrhythmia caused by QT time prolongation. During drug development, hERG channel interactions are determined very early to avoid heart problems. In the field of σ_1 receptors the hERG channel is of particular importance, since the pharmacophores of σ_1 receptor ligands and hERG channel blockers are very similar [51]. In the mouse capsaicin assay, **11** showed high analgesic activity, which is in the same range as the analgesic activity of S1RA (Fig. 4.1). Therefore, **11** is regarded as σ_1 receptor antagonist. Incubation with rat liver microsomes led to the identification of seven metabolites. The acetalic functional group represents a major position for metabolic transformations [50].

In order to remove the chemically and metabolically labile acetalic functionality and to install a structural element, which allows the introduction of a fluorine atom at the very end of the synthesis, the methoxy group of **11** was replaced by homologous fluoroalkyl residues (compounds **12–15** in Fig. 4.5). The homologous fluoroalkyl derivatives **12–15** show low nanomolar up to subnanomolar σ_1 affinity (see Fig. 4.5). Moreover, all four homologs display very high subtype selectivity [52–57]. The fluoroethyl derivative **13**, which was termed fluspidine, showed the highest σ_1 affinity (K_i = 0.59 nM) and the highest subtype selectivity (1331-fold) of this series of compounds.

4.3.2 Radiosynthesis of [^{18}F]12-[^{18}F]15

Due to the high σ_1 affinity all four fluoroalkyl derivatives **12–15** were synthesized in radioactive form (Scheme 4.1). For this purpose the tosylates **16–19** were reacted with K[^{18}F]F complexed with the cryptand K2.2.2. (Kryptofix®). The higher homologs [^{18}F]**13**-[^{18}F]**15** were obtained in high radiochemical yield and purity within 20–30 min in refluxing acetonitrile [47, 55, 57]. However, the fluorination of the tosyloxymethyl derivative **16** required 20 min heating in DMSO at 150 °C to yield [^{18}F]**12** [52].

The complete procedure for the radiosynthesis, purification and formulation of the PET tracers [^{18}F]**12**-[^{18}F]**15** usually took less than 120 min (approx. 1 half-life of 18-fluorine). The radiochemical yield was in the range of 40–50 % and the radiochemical purity of the final PET tracers was >98.6 %.

Due to the high σ_1 affinity and convenient radiosynthesis the four PET tracers [^{18}F]**12**-[^{18}F]**15** were evaluated *in vivo*. Although all four PET tracers [^{18}F]**12**-[^{18}F]**15** were suitable for imaging of σ_1 receptors in the brain, the fluoroethyl derivative fluspidine (**13**) appeared to be

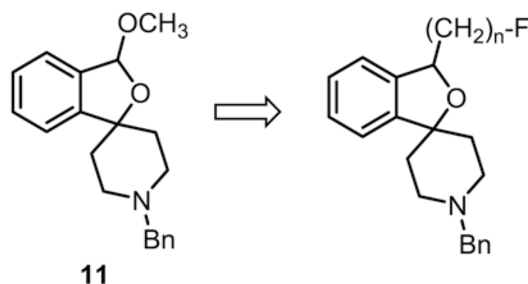
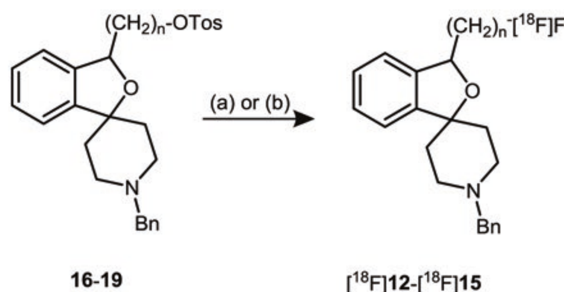


Fig. 4.5 Design of fluoroalkyl substituted spirocyclic 2-benzofurans **12**–**15** starting from the methoxy derivative **11**

compd.	n	$K_i(\sigma_1)$ (nM)	$K_i(\sigma_2)$ (nM)	selectivity
12	1	0.74	550	743
13	2	0.59	785	1331
14	3	1.4	837	598
15	4	1.2	489	408

the most promising candidate in terms of radiochemical availability, brain uptake (4.7 % ID/g, 30 min post injection (p.i.)), brain-to-plasma ratio (13, 60 min p.i.), specific binding (specific displacement by haloperidol), formation of radiometabolites (94 % of parent compound in plasma



compd.	n	solvent	T (°C)	t (min)	proced. time (min)[a]	rad. yield (%)[b]	purity (%)	spec. activ. (GBq/µmol)[c]
16 → 12	1	DMSO	150	20	90-120	38-50	>99.1	173-412
17 → 13	2	CH_3CN	85	25	90-120	35-45	>99.6	150-350
18 → 14	3	CH_3CN	85	30	90-120	35-48	>99.5	150-238
19 → 15	4	CH_3CN	83	20	90-120	45-51	>98.6	201-528

Scheme 4.1 Radiosynthesis of [^{18}F]**12**-[^{18}F]**15**
Reagents and reaction conditions: (a) K[^{18}F]F, K2.2.2., DMSO, K_2CO_3, 150 °C, 20 min, for [^{18}F]**12;** (b) K[^{18}F]F, K2.2.2., acetonitrile, K_2CO_3, 85 °C, 20–30 min for [^{18}F]**13**-[^{18}F]**15**

[a] procedure time: time for the overall procedure including synthesis, purification and formulation
[b] rad. yield: radiochemical yield, decay corrected
[c] spec. activ.: specific activity of the final PET tracer

30 min p.i., only one major radiometabolite was detected) and imaging contrast [54]. Therefore, the enantiomers of fluspidine (**13**) were separated before further studies were performed *in vivo*.

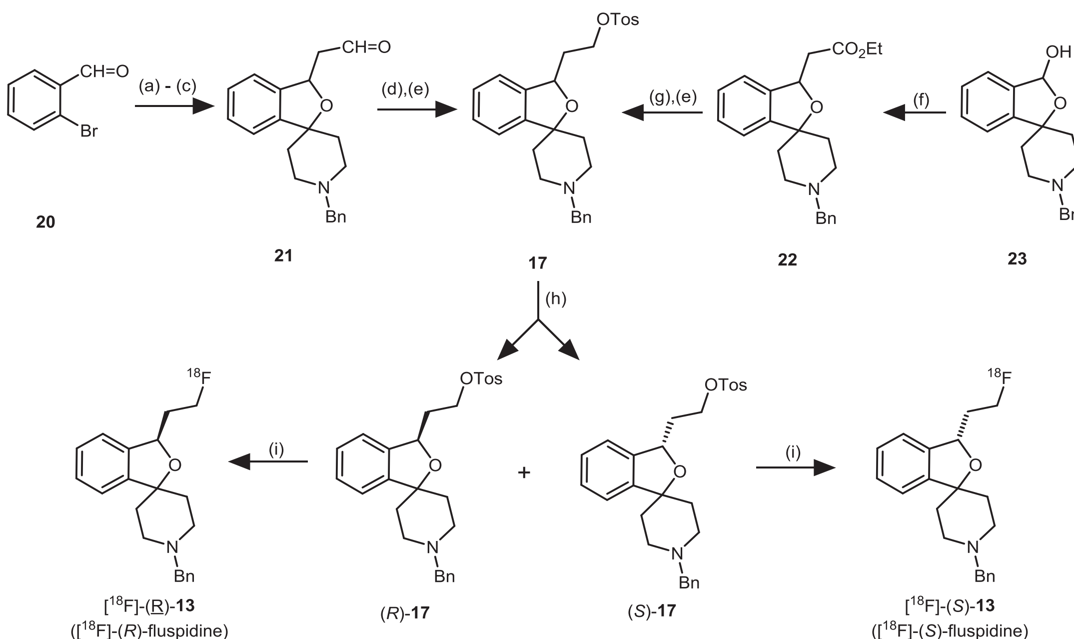
4.3.3 Synthesis of Enantiomerically Pure [^{18}F]-(*R*)- and -(*S*)-Fluspidine

For the synthesis of racemic fluspidine two independent routes are reported (Scheme 4.2). According to the first route, 2-bromobenzaldehyde was reacted with the *Wittig* reagent [(1,3-dioxolan-2-yl)methyltriphenylphosphonium bromide and K_2CO_3 to afford an α, β-unsaturated acetal [54]. Halogen metal exchange with *n*-BuLi, subsequent addition of the aryllithium intermediate at 1-benzylpiperidin-4-one and treatment of the product with HCl gave the aldehyde **21**. Reduction of the aldehyde **21** with $NaBH_4$ led to an alcohol, which was converted directly into the fluoroethyl derivative **13** upon treatment with DAST (diethylaminosulfur trifluoride). For the radiosynthesis of enantiomerically pure fluspidine enantiomers the tosylate **17** represented the central intermediate.

In an alternative route, the tosylate **17** was obtained starting with the hemi acetal **23**. The P-ylide $Ph_3P{=}CHCO_2Et$ reacted with **23** in a *Domino* reaction, consisting of ring opening of the hemi acetal **23** to give an hydroxyaldehyde, *Wittig* reaction of the aldehyde with the P-ylide and subsequent intramolecular conjugate addition to the α,β-unsaturated ester [49]. Reduction of the ester **22** with $LiAlH_4$ provided the alcohol, which was transformed into the tosylate **17**.

Reagents and conditions: (a) [(1,3-dioxolan-2-yl)methyltriphenylphosphonium bromide, $[(CH_2O)_2CHCH_2PPh_3\ Br]$, K_2CO_3, tris[methoxyethoxyethyl)amine. (b) *n*-BuLi, −78 °C, 1-benzylpiperidin-4-one. (c) HCl, THF. (d) $NaBH_4$, CH_3OH. (e) TosCl, NEt_3, DMAP. (f) $Ph_3P{=}CHCO_2Et$, Cs_2CO_3, toluene, reflux. (g) $LiAlH_4$, Et_2O, −15 °C. (h) chiral preparative HPLC, Daicel Chiralpak IB®. (i) K[^{18}F]F/K2.2.2, K_2CO_3, CH_3CN, 85 °C.

The enantiomeric tosylates (*R*)-**17** and (*S*)-**17** were separated by a chiral preparative HPLC using a Daicel Chiralpak IB® column [58]. The enantiomeric tosylates (*R*)-**17** and (*S*)-**17** were isolated in 98.2 % ee and 97.8 % ee, respectively. Reaction of the enantiomeric tosylates (*R*)-**17** and (*S*)-**17** with tetrabutylammonium fluoride



Scheme 4.2 Synthesis of enantiomerically pure (*R*)- and (*S*)-fluspidine (*R*)-**13** and (*S*)-**13**

(TBAF) in THF resulted in the fluspidine enantiomers (*R*)-**13** (99.6 % ee) and (*S*)-**13** (96.4 % ee). The absolute configuration was determined by circular dichroism. The σ_1 affinity of the fluspidine enantiomers (*R*)-**13** and (*S*)-**13** was 0.57 nM and 2.3 nM indicating the (*R*)-enantiomer being the eutomer with an eudismic ratio of 4.

Due to the high σ_1 affinity of both fluspidine enantiomers (*R*)-**13** and (*S*)-**13**, the radiosynthesis of both [^{18}F](*R*)-**13** and [^{18}F](*S*)-**13** was performed by nucleophilic substitution of the tosylates (*R*)-**17** and (*S*)-**17** with K[^{18}F]F complexed with the cryptand Kryptofix$^{(R)}$. For the careful preclinical evaluation of the PET tracer fluspidine [^{18}F]**13** and its enantiomers [^{18}F](*R*)-**13** and [^{18}F](*S*)-**13** an automated radiosynthesis was developed [59]. The key features of the automated radiosynthesis are a reaction time of 15 min in boiling acetonitrile (85 °C), 59 ± 4 min time for the complete process, 37 % radiochemical yield (decay corrected) and >98.8 % radiochemical purity.

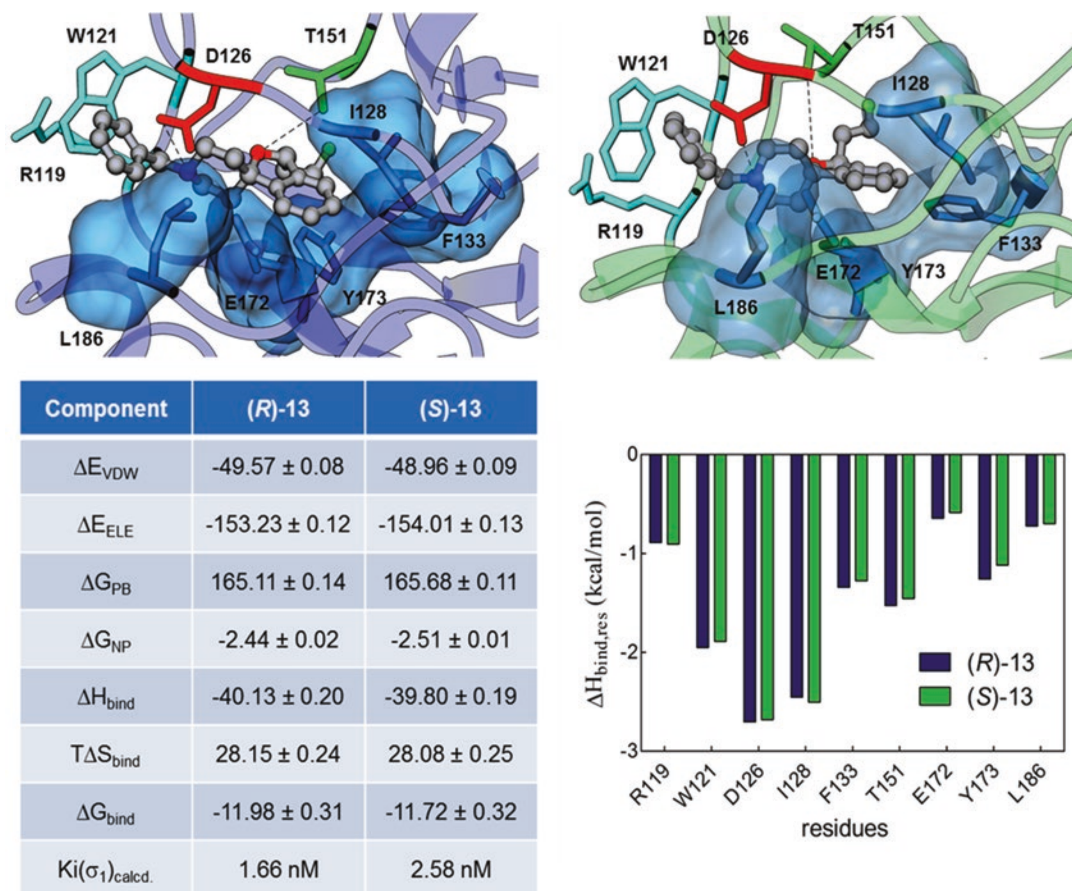
4.3.4 Interaction of (*R*)- and (*S*)-Fluspidine with the σ_1 Receptor

For a molecular-level description of the binding of fluspidine enantiomers to the σ_1 receptor, the putative binding modes of (*R*)-**13** and (*S*)-**13** on our σ_1 receptor 3D homology model were retrieved [60–62]. The two enantiomers were then docked into the putative binding site of the σ_1 receptor, and their affinity toward the receptor was scored after long runs of Molecular Dynamics (MD) simulation by MM/PBSA (Molecular Mechanics/Poisson Boltzmann Surface Area) analysis [63].

In a typical structure of the MD-simulated σ_1–ligand complexes, both (*R*)-**13** and (*S*)-**13** are oriented horizontally inside the receptor binding pocket and adopt similar binding poses, as illustrated in Fig. 4.6. For both enantiomers, the -NH$^+$ moiety of the ligand piperidine ring is anchored around the negatively charged side chain of D126 of the σ_1 protein, interacting with each other through a permanent salt bridge. As tracked by MD simulations, the average distance for the salt bridge through the proton at the cationic moiety of **13** and the COO$^-$ group of σ_1 D126 is 3.1 ± 0.1 Å for the (*R*)-enantiomer and 3.3 ± 0.1 Å for the (*S*)-enantiomer (Fig. 4.6, upper panels). A stable hydrogen bond between the donor hydroxyl group of T151 and the acceptor counterpart in the benzofuran moiety of fluspidine is also detected during the entire course of the MD simulation. Of note, the hydrophobic pocket lined by the side chains of the receptor residues I128, F133, Y173 and L186 with the further stabilizing contribution of E172 perfectly encases the 2-benzofuran portion of both enantiomers. Finally, the equilibrated MD trajectories revealed the presence of stabilizing π/cation and π/π interactions between the N-benzyl ring of (R)-**13** and (S)-**13** and the side chains of R119 and W121.

All of the interactions described above are quantified by a calculated free energy of binding ΔG_{bind} (Fig. 4.6, bottom left panel) for σ_1 equal to −11.98 ± 0.31 kcal/mol for (R)-**13** and −11.72 ± 0.32 for (S)-**13** corresponding to an estimated affinity $K_i(\sigma_1)_{calcd.}$ Value of 1.66 nM and 2.58 nM, respectively, in stringent agreement with the experimentally determined K_i values.

Analyzing the single energetic component of the binding free energy, we can see that for both compounds the overall polar component disfavors binding (i.e., $\Delta E_{ELE} + \Delta G_{PB}$ = +11.88 kcal/mol for (*R*)-**13** and +11.67 kcal/mol for (*S*)-**13**, respectively). However, the decomposition of the polar interactions into its Coulombic (ΔE_{ELE}) and solvation contributions (ΔG_{PB}) shows that indeed the direct intermolecular electrostatic interactions (ΔE_{ele}) are always favorable to binding but their contribution cannot compensate the large, unfavorable term (ΔG_{PB}) stemming from desolvation penalties associated with the binding event, thereby ultimately leading to an unfavorable contribution. In contrast, the intermolecular van der Waals interactions (ΔE_{VDW}) and the nonpolar solvation term (ΔG_{NP}) provide the driving force for binding. The highly favorable total nonpolar binding free energy reproduces the considerable contribution afforded by the stabilizing interactions from the various hydrophobic residues that line the surface of the binding cavity between the receptor and the ligands (i.e., $\Delta E_{VDW} + \Delta G_{NP}$ = −52.01 kcal/mol for (*R*)-**13** and −51.47 kcal/mol for (*S*)-**13**, respectively).



Component	(*R*)-13	(*S*)-13
ΔE_{VDW}	-49.57 ± 0.08	-48.96 ± 0.09
ΔE_{ELE}	-153.23 ± 0.12	-154.01 ± 0.13
ΔG_{PB}	165.11 ± 0.14	165.68 ± 0.11
ΔG_{NP}	-2.44 ± 0.02	-2.51 ± 0.01
ΔH_{bind}	-40.13 ± 0.20	-39.80 ± 0.19
$T\Delta S_{bind}$	28.15 ± 0.24	28.08 ± 0.25
ΔG_{bind}	-11.98 ± 0.31	-11.72 ± 0.32
$Ki(\sigma_1)_{calcd.}$	1.66 nM	2.58 nM

Fig. 4.6 Details of the key interactions detected in the equilibrated MD snapshots of (*R*)-**13** (*upper left panel*) and (*S*)-**13** (*upper right panel*) in complex with the σ_1 receptor. The main protein residues involved in ligand/receptor interactions are R119, W121 (π-interaction; *cyan*), D126 (salt bridge; *red*), I128, F133, E172, Y173, L186 (hydrophobic cavity; steel *blue*) and T151 (hydrogen bond; *green*). Compounds (*R*)-**13** and (*S*)-**13** are shown in atom-colored sticks and-balls: C, *gray*; O, *red*; and N, *blue*. H atoms are not shown but the salt bridges and the hydrogen bonds are indicated as black broken lines. In both panels, water molecules, ions and counterions are not shown for clarity. (*bottom left panel*) Binding free energy (ΔG_{bind}) and its components for (*R*)-**13** and (*S*)-**13** in complex with the σ_1 receptor. All energy values are in kcal/mol. The calculated $Ki(\sigma_1)_{calcd.}$ Values (nM), obtained using the relationship $\Delta G = -RTln(1/K_i)$ are also reported. (*bottom right panel*) Per residue enthalpic contribution ($\Delta H_{bind,res}$) to binding for the σ_1 receptor in complex with (*R*)-**13** and (*S*)-**13**. Only σ_1 residues critical for receptor binding are shown

Finally, the entropic terms for both enantiomers are almost equal (i.e., $T\Delta S_{bind}$ = +28.15 kcal/mol for (*R*)-**13** and +28.08 kcal/mol for (*S*)-**13**, respectively), as could be expected since the two molecules are identical both from the standpoint of the molecular structure and the loss in degrees of freedom they undergo upon binding to the σ_1 receptor. As illustrated in the bottom right panel of Fig. 4.6, a deconvolution of the enthalpic component ($\Delta H_{bind,res}$) of the binding free energy into contributions from each protein residue was carried out to investigate in detail the binding mode of both enantiomers (*R*)-**13** and (*S*)-**13** to the σ_1 receptor. Specifically, the stable hydrogen bond involving T151 (Average Dynamic Length (ADL) = 2.00 ± 0.02 Å for (*R*)-**13** and 2.06 ± 0.02 Å for (*S*)-**13**) and the stable salt bridge featured by D126 are responsible for stabilizing contributions of −1.53 kcal/mol for (*R*)-**13** and −1.46 kcal/mol for (*S*)-**13** and −2.70 kcal/mol for (*R*)-**13** and −2.68 kcal/mol for

(*S*)-**13**, respectively. Moreover, substantial van der Waals and electrostatic interactions are contributed by residues R119 (−0.89 kcal/mol for (*R*)-**13** and −0.90 kcal/mol for (*S*)-**13**) and W121 (−1.95 kcal/mol for (*R*)-**13** and −1.89 kcal/mol for (*S*)-**13**), through the aforementioned π-cation and T-stacking π−π interaction, respectively, and by the residues belonging to the hydrophobic pocket I128, F133, E172, Y173, L186 ($\sum \Delta H_{bind}$,res = −6.42 kcal/mol for (*R*)-**13** and $\sum \Delta H_{bind}$,res = −6.19 kcal/mol for (*S*)-**13**). All other receptor residues were characterized by negligible interaction enthalpy values ($|\Delta H_{bind,res}| < 0.30$ kcal/mol).

4.3.5 *In Vitro* Biotransformation of (*R*)- and -(*S*)-Fluspidine

The biotransformation was investigated *in vitro* upon incubation of both non-labeled fluspidine enantiomers (*R*)-**13** and (*S*)-**13** with rat liver microsomes and NADPH/H^+. At first the rate of degradation was determined in kinetic experiments over a period of 90 min[57]. After an incubation period of 30 min approx. 72 % of both parent fluspidine enantiomers were detected. However, after a period of 90 min, only 33 % of (*R*)-**13** was left, whereas 58 % of unchanged (*S*)-**13** was found. These experiments indicate a higher metabolic stability of (*S*)-fluspidine (*S*)-**13** compared to its (*R*)-configured enantiomer (*R*)-**13**.

In the next step, the number and structure of the formed metabolites was analyzed [58]. For (*S*)-fluspidine (*S*)-**13** eight metabolites were identified by LC-MS experiments: the N-debenzylated metabolite (*S*)-**13A**, four monooxygenated metabolites (*S*)-**13B–E** and three metabolites containing two additional O-atoms. In Fig. 4.7 the primary metabolites of (*S*)-**13** are depicted showing the metabolic labile positions, which are attacked by CYP enzymes. Regarding the biotransformation the N-benzyl moiety represents a privileged structural element for degradation, since the N-debenzylated metabolite (*S*)-**13A**, the 4-hydroxybenzyl metabolite (*S*)-**13B** and the

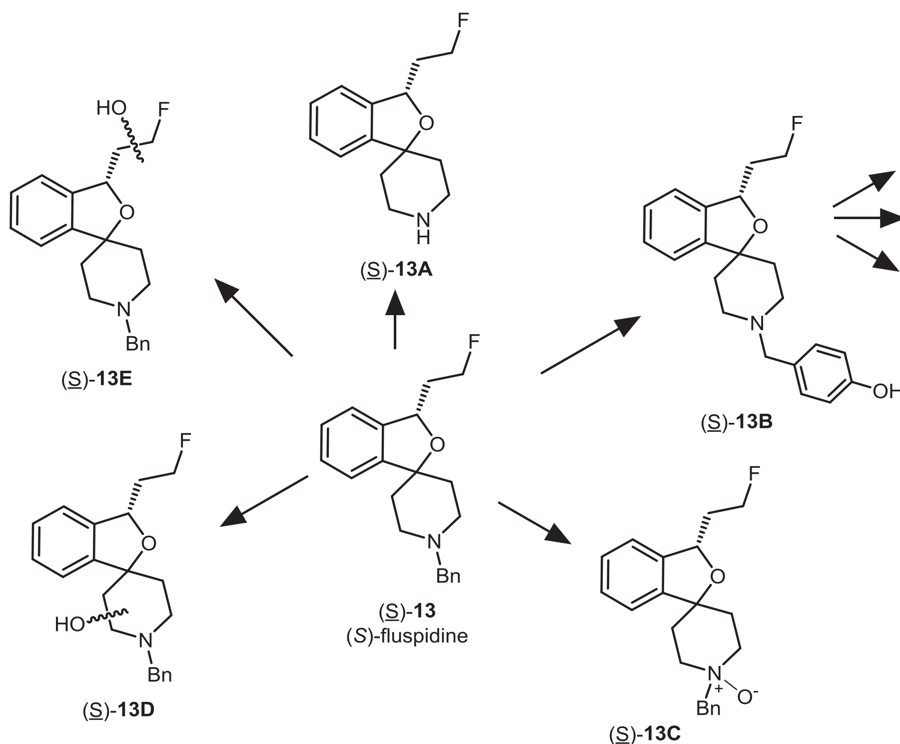


Fig. 4.7 Metabolically labile positions of (*S*)-fluspidine (*S*)-**13**

N-oxide (*S*)-**13C** were identified. Additionally, metabolites with an OH moiety at the piperidine ring ((*S*)-**13D**) and at the fluoroethyl side chain ((*S*)-**13E**) were identified. The dioxygenated metabolites were formed by further hydroxylation of the 4-hydroxybenzyl metabolite (*S*)-**13B**. at the piperidine ring, the N-atom, and the hydroxybenzyl moiety. Although (*R*)-**13** was transformed faster than (*S*)-**13**, only seven metabolites were formed upon incubation of (*R*)-**13** with rat liver microsomes and NADPH/H^+. The metabolite (*R*)-**13E** bearing the OH moiety in the fluoroethyl side chain could not be detected for the enantiomer (*R*)-**13**.

4.3.6 *In Vivo* PET Studies with [^{18}F](*R*)- and [^{18}F](*S*)-Fluspidine

The *in vivo* kinetics was carefully investigated after application of the radiotracers [^{18}F](*S*)-**13** and [^{18}F](*R*)-**13** to mice and piglets [64]. In the piglet study the brain uptake and wash-out kinetics of the enantiomers [^{18}F](*S*)-**13** and [^{18}F](*R*)-**13** showed significant differences. The initial uptake of the enantiomers was very similar, but the wash-out of [^{18}F](*R*)-**13** was very slow. After 120 min the concentration of [^{18}F](*R*)-**13** in the brain was almost the same as after 5 min indicating a quasi-irreversible but non-covalent binding of the more affine enantiomer [^{18}F](*R*)-**13**. The specific interactions of [^{18}F](*S*)-**13** and [^{18}F](*R*)-**13** with σ_1 receptors was reduced by administration of the selective σ_1 ligand SA4503 confirming the selective labeling of σ_1 receptors (Fig. 4.8, part c). The recorded data were used to establish a tracer kinetic model for both enantiomers, the influx rate constant k_1, the clearance rate constant k_2', and the binding potential (k_3'/k_4) for various brain regions were estimated and the distribution volume in the whole brain was calculated.

Determination of the plasma concentration of the parent compounds showed that the metabolic degradation of [^{18}F](*R*)-**13** is significantly faster than that of [^{18}F](*S*)-**13**, which confirms the *in vitro* experiment with rat liver microsomes. For both enantiomers lipophilic radiometabolites, which would be able to penetrate into the brain, were not detected.

In Fig. 4.8 brain images obtained after treatment of the animals with the fluorinated PET tracer [^{18}F](*S*)-fluspidine ([^{18}F](*S*)-**13**) are shown. The *ex vivo* autoradiography of a mouse brain (Fig. 4.8 part a) reveals high concentration of the radioligand in those regions, which are reported to be rich in σ_1 receptors. The resolution of the PET image of the whole piglet brain (Fig. 4.8 part b) is reduced compared to the mouse autoradiography, which directly detects β^+ (e^+) particles, whereas PET detects gamma ray coincidences. The specificity of [^{18}F](*S*)-fluspidine binding to σ_1 receptors was investigated with a blocking experiment. In which a large amount of SA4503 was administered. In Fig. 4.8, part c, the thalamus of piglet brain is displayed exemplarily indicating that labeling with [^{18}F](*S*)-fluspidine can be inhibited by the σ_1 receptor ligand SA4503

4.4 Conclusion

Preclinical evaluation performed with [^{18}F](*S*)- and [^{18}F](*R*)-fluspidine ([^{18}F](*S*)-**13**) and ([^{18}F](*R*)-**13**) indicates that both tracers are valuable tools for selective non-invasive visualization and quantification of σ_1 receptors in the brain under healthy and diseased conditions. To provide a molecular rationale of the interactions between fluspidine enantiomers and the σ_1 receptor, the two enantiomers were docked into the putative binding site of our σ_1 3D receptor model, and their affinity toward the receptor was scored by MM/PBSA analysis. The results of our modeling investigations confirm that both enantiomers of **13** can be accommodated within the σ_1 binding site and establish the same network of stabilizing interactions with the target receptor. Although (*S*)-fluspidine shows lower σ_1 affinity than (*R*)-fluspidine, the higher metabolic stability of (*S*)-fluspidine and its common reversible binding kinetics favors slightly this enantiomer for further development. However, the potential of the non-covalent quasi-irreversible σ_1 binding of [^{18}F](*R*)-fluspidine remains to be further elucidated in clinical studies.

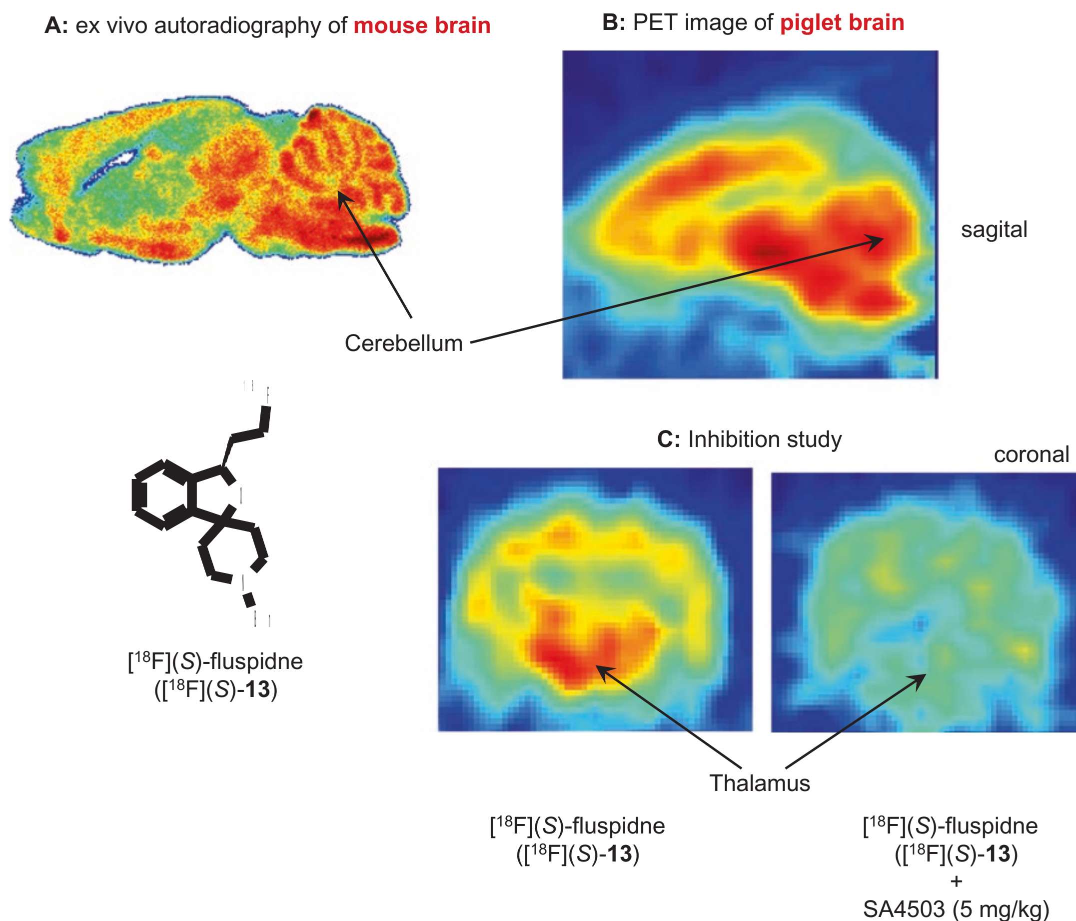


Fig. 4.8 Studies with the PET tracer $[^{18}F](S)$-fluspidine ($[^{18}F](S)$-**13**): (**a**): *ex vivo* autoradiography of mouse brain; (**b**): PET image of whole piglet brain; (**c**): inhibition study with the σ_1 ligand SA4503; the region of the thalamus is shown exemplarily (Modified according to Ref. [2])

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The Evolution of the Sigma-2 (σ_2) Receptor from Obscure Binding Site to *Bona Fide* Therapeutic Target

5

Chenbo Zeng and Robert H. Mach

Abstract

The sigma-2 (σ_2) receptor represents one of the most poorly understood proteins in cell biology. Although this receptor was identified through in vitro binding studies over 25 years ago, the molecular identity of this protein is currently not unambiguously known, and the results from recent attempts to identify the σ_2 receptor through protein purification and mass spectral analysis have been the subject of debate in the literature. However, there is overwhelming data demonstrating that the σ_2 receptor is an important biomarker of tumor cell proliferation. The observation that σ_2 receptor agonists are potent anticancer agents whereas σ_2 antagonists block Aβ1-42 oligomer synaptic dysfunction in transgenic mouse models of Alzheimer's disease have clearly identified this protein as an important therapeutic target for the treatment of a variety of pathological conditions.

Keywords

σ_2 receptors • Cell proliferation • Cancer chemotherapy • Agonists • Antagonists

5.1 Introduction: Identification and Characterization of the σ_2 Receptor

The sigma (σ) receptors are perhaps the most poorly understood protein in the field of cell biology today. Although they were initially identified over 40 years ago, and studied by numerous research groups around the world, the biological function of σ receptors in normal tissues and in cancer cells is still unknown [1]. The most enigmatic of the σ receptors is the σ_2 receptor subtype, and there is currently an ongoing debate in the literature regarding the most basic elements of the structure and function of this protein.

The existence of multiple σ receptors was initially postulated on data obtained from a series of in vitro binding studies conducted by Hellewell

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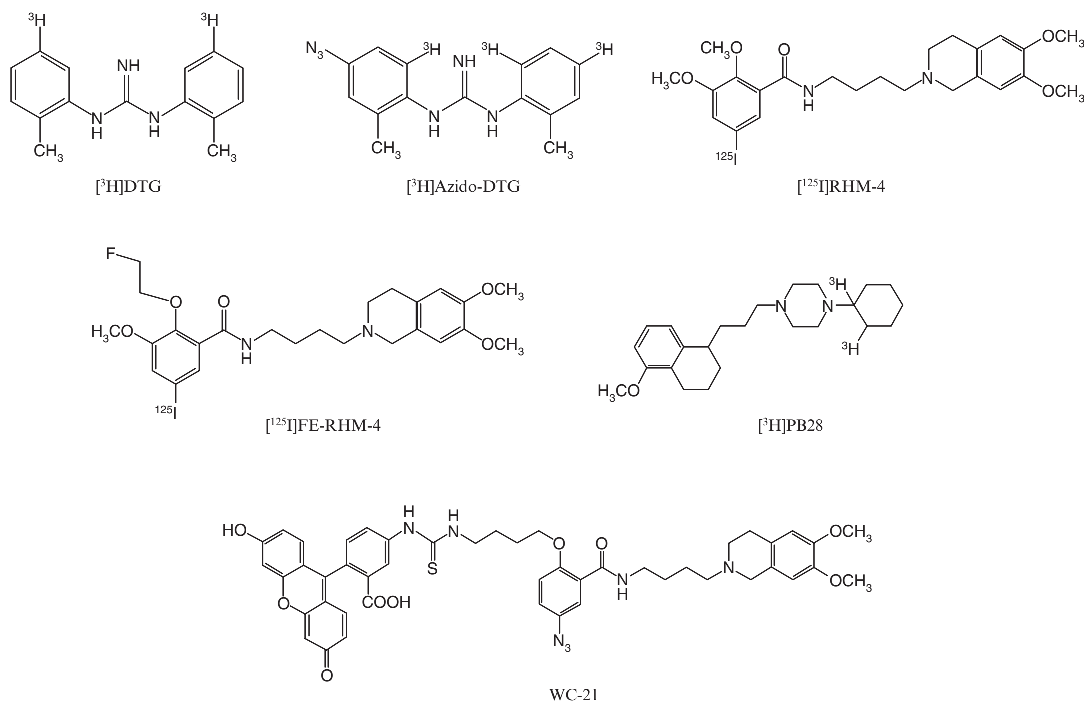

Fig. 5.1 Structures of radiolabeled probes and the photoaffinity probe used to characterize the σ_2 receptor

and Bowen [2, 3]. The σ ligands [³H]DTG (Fig. 5.1) and [³H](+)-pentazocine bound with high affinity to σ receptor sites in guinea pig brain, but the affinity of [³H](+)-pentazocine was 1000-fold lower than the affinity of [³H]DTG to σ binding sites in pheochromocytoma (PC12) cells. Furthermore, the receptor density (i.e., B_{max}) values of [³H](+)-pentazocine were only 25 % that of the density of σ receptors measured with [³H] DTG. These data suggested the existence of multiple σ receptor binding sites, and was later confirmed by photoaffinity labeling studies with the radiolabeled probe, [³H]azido-DTG (Fig. 5.1). That is, [³H]azido-DTG labeled two protein bands in rat liver membrane homogenates: a 25 kDa protein whose photoaffinity labeling could be blocked with the benzomorphan, dextrallorphan; and a 21.5 kDa protein whose labeling by [³H]azido-DTG could not be blocked by dextrallorphan [3]. The 25-kDa band was named the σ_1 receptor, and the 21.5 kDa band was termed the σ_2 receptor. When similar photoaffinity labeling studies were conducted in PC12 cells, the 25 kDa band was not present whereas two bands corresponding to the σ_2 receptor having molecular weights (MW) of 21.5 and 18 kDa were observed [2]. Consequently, the σ_2 receptor is often described in the literature as having a MW of 18–21.5 kDa.

Although this biochemical characterization of the σ_2 receptor was reported over 25 years ago, the molecular identity of the σ_2 receptor is currently unknown since this protein has not been purified, sequenced or cloned. There have been a number of attempts to identify the σ_2 receptor by purifying and sequencing the protein using mass spectral (MS) analysis. Colabufo et al. [4] used an analog of the σ_2 receptor ligand PB28coupled to an affinity column to enrich the protein. The trapped proteins were eluted from the column, separated by SDS-PAGE gel electrophoresis, and characterized using MALDI and LC-MS analysis. This procedure identified six histone-binding proteins as potential PB28-binding proteins. The radioligand, [³H]**PB28** (Fig. 5.1), was then shown to bind to a reconstituted histone H2A/H2B dimer, leading the authors to hypothesize [5] that the σ_2 receptor is either a histone-binding protein, or that σ_2 receptors bind to histone proteins as an

additional site within the cell where σ_2 receptors are localized. However, the lack of similarity between the subcellular localization of histone proteins (in the nucleus of the cell) versus σ_2 receptors, which are located on the plasma membrane, endoplasmic reticulum and mitochondria, make this hypothesis unlikely. [6, 7].

More recently, Xu et al. used the FITC-tagged photoaffinity agent, **WC-21** (Fig. 5.1), to label the σ_2 receptors expressed in rat liver membrane homogenates. [8] Western blot analysis revealed a dominant protein band at ~24 kDa that was labeled by FITC-conjugated probe **WC-21**. Labeling of this protein band with **WC-21** was blocked by well-characterized σ_2 receptor ligands. MS and proteomic studies identified this 24 kDa protein as the *progesterone receptor membrane component 1* (PGRMC1). [8, 9] The primary amino acid sequence of the PGRMC1 has a MW of 21.5 kDa, which is identical to the 21.5 kDa MW of the σ_2 receptor in rat liver membranes reported by Hellewell and Bowen. [3] Radioligand binding studies were conducted using [^{125}I]**FE-RHM-4**, an analog of the radioiodinated sigma-2 receptor ligand [^{125}I]**RHM-4** (Fig. 5.1) in cells either having a transient knockdown or overexpression of PGRMC1. Transient knockdown of PGRMC1 using a PGRMC1-specific siRNA decreased the binding of [^{125}I]**FE-RHM-4** to HeLa cells, whereas overexpression of PGRMC1 led to an increase in the binding of [^{125}I]**FE-RHM-4** in these cells. These data indicate that the PGRMC1 complex has binding properties similar to the σ_2 receptor. In addition, the similar subcellular localization of the PGRMC1 and σ_2 receptors in HeLa cells, and the association of both the σ_2 receptor and PGRMC1 with cytochrome P450 proteins provided additional evidence supporting a functional link between proteins. Based on the above data, the authors concluded that the "putative σ_2 receptor" represented a binding site located within the PGRMC1 protein complex. [8] However, recent studies using stable cell lines where the PGRMC1 had been knocked out using either a PGRMC1 shRNA or PGRMC1 CRISPR vector has shown that there is no decrease in binding of [^{3}H]DTG binding in PGRMC1 knockout cells. [10, 11] These data indicate that the DTG-sensitive σ_2 receptor-binding site is not located within the amino acid sequence of the PGRMC1, and has raised questions regarding the validity of the conclusions reported by Xu et al. [8].

5.2 σ_2 Receptor Ligands as Potential Cancer Therapeutic Drugs

Although the molecular identity of the σ_2 receptor is still unclear, there is overwhelming evidence from in vitro binding and pharmacology studies demonstrating that the σ_2 receptor is an important biomarker of cell proliferation in cancer cells. For example, σ_2 receptors are expressed in high density in nearly all human and rodent tumor cell lines [12], and the density of σ_2 receptors has been correlated with the grade of some human and bovine tumors [13–15]. The density of σ_2 receptors in proliferating mouse mammary adenocarcinoma cells was found to be ~tenfold higher than that in the corresponding quiescent tumor cells [16, 17]. These observations have led to the development of the σ_2 receptor ligands not only as molecular probes for the imaging of solid tumors, but also as potential therapeutic agents for the treatment of cancer [18].

5.2.1 σ_2 Receptor Ligands as Potential Single Agent Drugs

Bowen et al. reported that the σ_2 ligands **CB-64D** and **CB-184** (Fig. 5.2) induced caspase-independent apoptosis in human breast cancer cells such as MCF-7 [19]. Various σ_2 ligands including **CB-64D** produced an immediate and transient calcium release in SK-N-SH neuroblastoma cells [20]. The transient rise in calcium ions was blocked by thapsigargin, a sesquiterpene lactone that is a non-competitive inhibitor of the endoplasmic reticulum Ca^{2+} ATPase, suggesting that the calcium ions were released from the endoplasmic reticulum. In another study, this group reported that low concentrations of

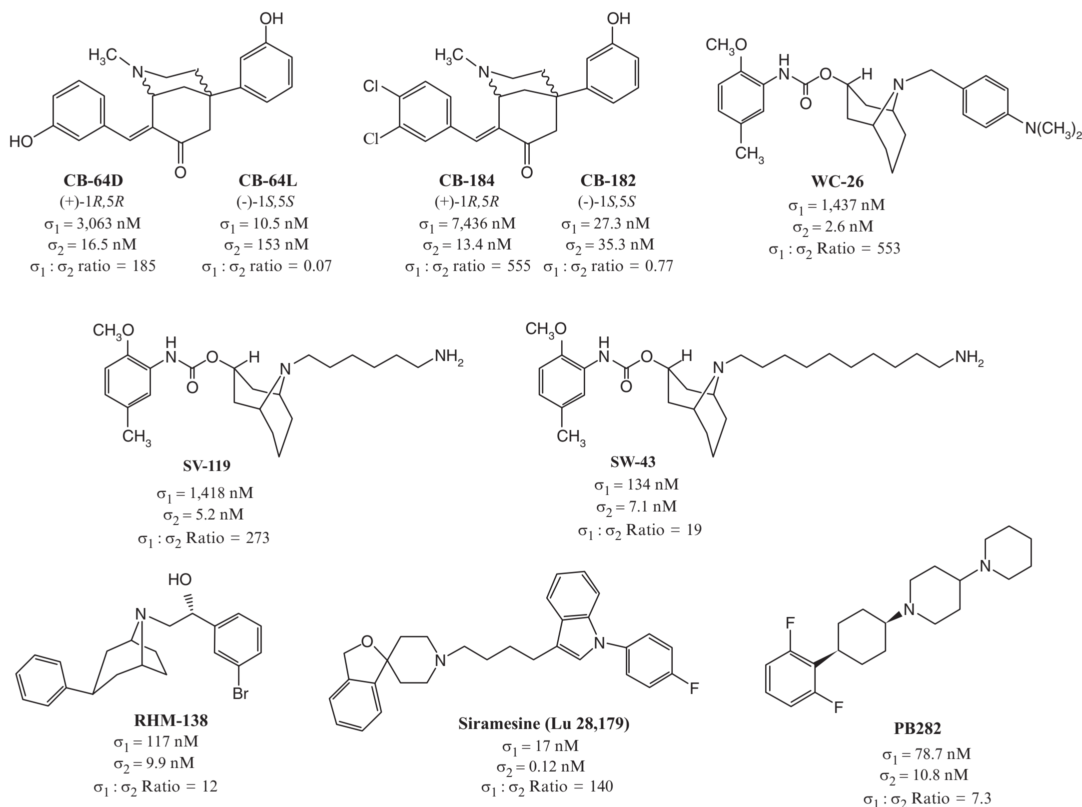

Fig. 5.2 Structures of σ_2 receptor agonists

CM572, a partial agonist at σ_2 receptors, significantly attenuated the calcium signal and cell death induced by the σ_2 agonist **CB-64D** [21]. Since dysregulated calcium homeostasis in cells can cause cell death, these data suggest that σ_2 ligands may induce cytotoxicity through endoplasmic reticulum and calcium release. Moreover, the σ_2 ligand **CB-184** (10 μM) caused an increase in ceramide and concomitant decrease in sphingomyelin in breast cancer cells [22]. Ceramide is a sphingolipid second messenger involved in cell proliferation. Ceramide can either promote cell proliferation or induce apoptosis depending on the cell type. The data suggest that σ_2 receptors may produce effects on cell growth and apoptosis by regulating the sphingolipid pathway.

Mach et al. has previously reported the synthesis and *in vitro* characterization of a number of structurally diverse ligands with a high affinity for σ_2 receptors [23, 24]. By screening these sigma ligands for their cytotoxicity, some σ_2 receptor ligands were found to possess potent cytotoxic activities for various cancer cells including mouse breast cancer EMT-6 and human MDA-MB-435 melanoma tumor cells, the EC_{50} values in the cytotoxicity assay were in the micromolar range [25]. Normalizing the cytotoxicity of a σ_2 ligand relative to that of siramesine, a commonly-accepted σ_2 agonist, it was possible to categorize these σ_2 receptor ligands into agonists, partial agonists, and antagonists [26]. Three ligands (**WC-26**, **SV119** and **RHM-138**) (Fig. 5.2) induced DNA fragmentation and caspase-3 activation. The caspase inhibitor Z-VAD-FMK partially blocked the DNA fragmentation and cytotoxicity caused by these compounds. These data suggest that the cell death induced by these σ_2 receptor ligands was caused, in part, by caspase-mediated apoptosis. **WC-26** also induced formation of vacuoles in the cells. **WC-26**, **SV119, RHM-138** increased the synthesis and processing of microtubule-associated protein

light chain 3 (LC3), an autophagosome marker, and decreased the expression levels of the downstream effectors of mammalian target of rapamycin (mTOR), p70S6K and 4EBP1, suggesting that σ_2 receptor ligands induce autophagy, probably by inhibition of the mTOR pathway. All three σ_2 receptor ligands decreased the expression of cyclin D1 in a time-dependent manner. In addition, **WC-26** and **SV119** decreased cyclin B1, E2 and phosphorylation of retinoblastoma protein (pRb). These data suggest that σ_2 receptor ligands also impair cell-cycle progression in multiple phases of the cell cycle.

Hawkins et al. showed that **SW43** (Fig. 5.2), an analogue of **SV119**, induced cell death in Bxpc3 and Aspc1 pancreatic cancer cells [27, 28]. **SW43** accumulated in lysosomes, caused lysosomal leakage and triggered apoptosis. Microscopy studies of **SW120** [29], a σ_2 receptor fluorescent probe structurally similar to **SW43** (Fig. 5.3), showed accumulation in the lysosomes of cancer cells. Since the cytotoxicity of **SW43** can be partially blocked by concanamycin A, an inhibitor of a V-Type ATPase H^+ pump, which increases lysosomal pH, lysosomal accumulation may be required for the cytotoxicity of this compound. **SW43** also increased levels of oxidative stress in the cell. The antioxidants N-acetylcysteine and α-tocopherol, but not the caspase-3 inhibitor DEVD-FMK, decreased **SW43**-induced cell death. These data suggest that **SW43** induced reactive oxygen species (ROS)-dependent and caspase-3-independent cell death in pancreatic cancer cells.

Siramesine (Fig. 5.2) is a σ_2 receptor ligand with a chemical structure different from that of **SW43**, **SV119**, or **WC26**. Several groups have investigated the anticancer mechanisms of siramesine. Jaattela et al. [30, 31] reported that siramesine (5 or 8 μM) rapidly accumulated in lysosomes, induced a rapid rise in the lysosomal pH, lysosomal leakage, cathepsin-dependent and caspase-3-independent cell death in various cancer cell lines including human breast cancer cell line MCF-7 and murine fibrosarcoma WEHI-S. Siramesine also triggered autophagosome formation and induced ROS while α-tocopherol inhibited siramesine-induced lysosomal pH rise, lysosomal leakage and cell death. These data appear to suggest that ROS may occur upstream of lysosomal pH rise and leakage. Turk et al. [32] reported that siramesine (20–45 μM) induced rapid loss of mitochondrial membrane potential (MMP), cytochrome c release, caspase

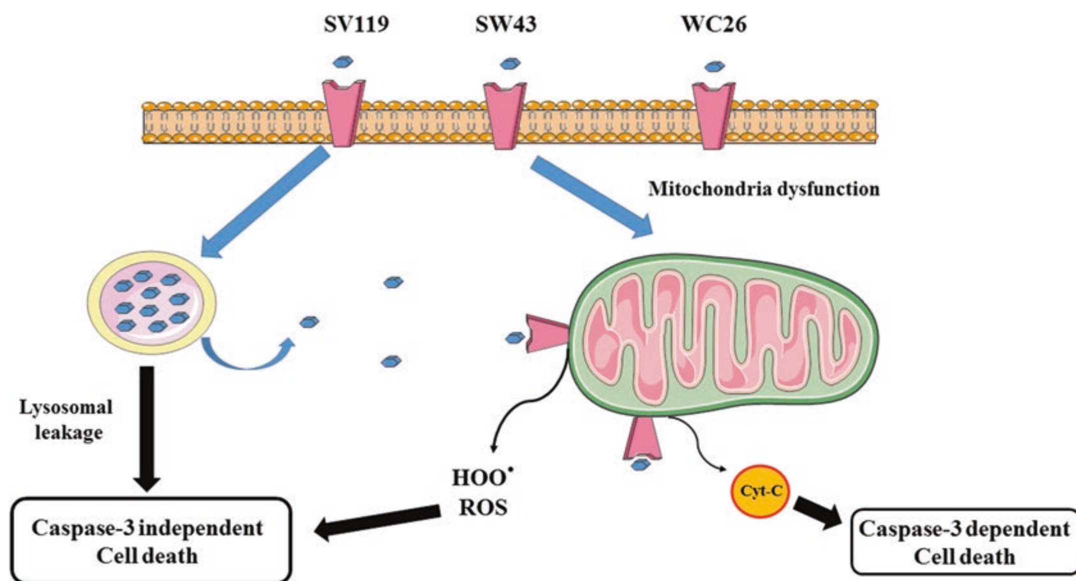

Fig. 5.3 Proposed mechanism of cell death induced by **SW43**, **SV119** and **WC26**

activation, and cell death in HaCaT cells, which are spontaneously immortalized human keratinocytes. The pancaspase inhibitor Z-VAD-FMK inhibited siramesine (20–45 μM)-induced cell death in HaCaT cells. Siramesine (20–40 μM) induced ROS and the antioxidant α-tocopherol reduced MMP loss, decreased ROS formation, significantly blocked cytochrome c release, caspase-3 activation and cell death. Siramesine (25–40 μM) also rapidly increased lysosomal pH, but did not cause lysosome leakage when measured for 2 h after treatment. These data suggest that, at concentrations above 20 μM, siramesine triggers cell death initially through the destabilization of mitochondria and subsequent activation of the intrinsic pathway of apoptosis. The data also suggest that siramesine-induced cell death may not be through the lysosomal leakage pathway in HaCaT cells. These results are different from the data reported by Jaattela et al., who demonstrated that siramesine induced lysosomal leakage and cathepsin-dependent cell death in MCF-7 cells. Whether or not lysosomal leakage is required for siramesine-induced cell death may be dependent on the cancer cell type.

SV119, **SW43** and **WC26** are 9-amino-[3.3.1] azabicyclononane or granatane analogs, which are structurally different from siramesine. Comparison of the mechanisms of cell death induced by these σ_2 receptor ligands reveals that there are certain similarities between the mechanisms of cell death induced by the two classes of compounds. Siramesine can partially block the uptake of a σ_2 fluorescent probe, **SW120**, into cells, suggesting that siramesine and **SW120** bind to the same site on the σ_2 receptor. **SW43** and siramesine both induce ROS, accumulate in lysosomes, form autophagosomes, and result in caspase-3-dependent and independent cell death. The antioxidant α-tocopherol inhibits cell death induced by **SW43** and siramesine. Therefore, we propose the following cell death mechanism for **SW43**, **SV119** and **WC-26** based on the data obtained: **SW43**, **SV119** and **WC-26** induce cell death through (1) lysosomal leakage and (2) mitochondria dysfunction. The σ_2 ligands rapidly accumulate in lysosomes/endosomes, increase lysosomal pH, cause lysosomal leakage, cathepsin-dependent and caspase-3-independent cell death. The σ_2 ligands also bind to mitochondria, cause mitochondria dysfunction, induce ROS and subsequently lead to caspase-3-independent cell death. In addition, σ_2 ligands induce cell death partially through caspase-3-dependent pathways, which may be involved in the mitochondria-mediated intrinsic pathway of apoptosis (Fig. 5.3). Whether the σ_2 ligands induce cell death through endoplasmic reticulum and calcium release needs to be determined.

PB282 (Fig. 5.2) also accumulates in lysosomes and induces lysosomal membrane permeabilization (LMP) in Bxpc3 and Aspc1 pancreatic cancer cells [27, 33, 34]. **PB282** did not induce ROS, although the antioxidants N-acetylcysteine and α-tocopherol provided protection from this ligand, suggesting that the protection by these antioxidants from **PB282**-induced cytotoxicity is through a mechanism other than inhibiting oxidative stress. The caspase-3 inhibitor DEVD-FMK significantly blocked **PB282**-induced cell death, and suggests that PB282 causes caspase-dependent cell death following LMP.

In an allogenic animal model of pancreatic cancer, Hawkins et al. showed that **WC-26** or **SV119** treatment activated caspase-3 activity in the tumor, decreased tumor growth, and increased the survival rate of tumor-bearing mice [35]. After a single dose of **WC-26** treatment, the mice appeared normal, caspase-3 activation in normal tissues was minimal, and blood chemistry was normal. The data suggest that there is minimal acute, systemic toxicity. In athymic nude mice inoculated subcutaneously with Bxpc3 cells, the σ_2 receptor ligands **SV119**, **SW43**, **PB28**, or **PB282** each significantly reduced tumor size [27].

The EC_{50} values of σ_2 receptor ligands for killing cancer cells are in the micromolar range, whereas their affinities (*Ki* values from in vitro binding studies) for σ_2 receptors are in nanomolar range. These observations raised the question whether the cytotoxicity of σ_2 receptor ligands is mediated by the σ_2 receptor. Bowen et al. showed that despite the requirement of micromolar concentrations of sigma ligands to induce morphological damages of C6 glioma cells, the effect was specific for sigma ligands, but not for various ligands of dopamine, serotonin, adrenergic, gluta-

mate, phencyclidine, GABA, opiate, or muscarinic cholinergic receptors [36]. These data suggest that the cytotoxicity of σ_2 receptor ligands is likely to be, at least in part, mediated by σ_2 receptors. In conclusion, σ_2 receptor ligands are potential single agent drugs for treating various cancers.

5.2.2 σ_2 Receptor Ligands as Potential Drug Delivery Agents for Cancer Cells

Zeng et al. previously demonstrated that the fluorescent σ_2 receptor probe, **SW120,** is rapidly internalized into cancer cells by receptor-mediated endocytosis and localizes in multiple subcellular organelles such as the mitochondria, lysosomes and endoplasmic reticulum [29, 37]. These data suggest that σ_2 receptor ligands are excellent candidates for delivering anticancer drugs selectively into tumors. This σ_2 receptor-targeting strategy has been used to deliver apoptosis-inducing peptides, the alpha-emitting radionuclide astatine-211 (At-211), and nanoparticles loaded with anticancer drugs to cancer cells in vitro and in vivo.

5.2.3 Delivery of Apoptosis-Inducing Peptides

In order to deliver death-inducing peptides into cancer cells, the σ_2 receptor ligand **SV119** was conjugated with two different polypeptides: (1) a 21 amino acid-containing the BH3 domain of the Bcl-2 antagonist Bim, and (2) a 25 amino acid peptide derived from an endogenous Akt-inhibitor, carboxyl terminal modular protein (CTMP) [38]. Both the Bim and CTMP peptides had been previously shown to induce cell death [35, 39]. Sigma-2-Bim and sigma-2-CTMP exhibited greater cytotoxicity in pancreatic cancer cells than either **SV119** or death inducing peptide alone under cell culture conditions. In murine models of pancreatic adenocarcinoma, sigma-2-Bim significantly reduced tumor size, and exhibited only limited normal tissue toxicity.

Second mitochondria-derived activator of caspase (Smac) is a protein released from the mitochondria into the cytosol, leading to apoptosis [40]. **SW43** has also been conjugated with a Smac mimetic compound (SMC), **SW-IV-52s**, to form **SW-III-123**. **SW-III-123** exhibited significantly greater cytotoxicity in human ovarian cancer cell lines than that by either **SW-IV-52s** or **SW43** alone and in combination, suggesting that targeting the σ_2 receptor successfully delivered SMC into ovarian cancer cells. **SW-III-123** induced rapid degradation of inhibitor of apoptosis proteins (cIAP1 and cIAP2), accumulation of NF-κB-inducing kinase (NIK) and phosphorylation of NF-κB p65, suggesting that **SW-III-123** activated both canonical and noncanonical NF-κB pathways in SKOV-3 cells. **SW-III-123** cleaved caspase-8, -9 and -3. An antibody for tumor necrosis factor alpha (TNFα) markedly blocked **SW-III-123**-induced cell death and caspase-3 activity in SKOV-3 cells, indicating that **SW-III-123** activated both the intrinsic and extrinsic pathways of apoptosis and induced TNFα-dependent cell death in SKOV-3 cells. Intraperitoneal administration of **SW-IV-134**, the biologically-active enantiomer of **SW-IV-123** (Fig. 5.4), significantly reduced tumor burden and improved overall survival in a mouse xenograft model of ovarian cancer without causing significant adverse effects to normal tissues [41]. Moreover, **SW-IV-134** has been shown to effectively kill pancreatic cancer cells in vitro and in vivo [42]. **SW-IV-134** also exhibited potent cytotoxicity to triple negative breast cancer cells [43]. Incubation cells with **SW-IV-134** for 2 h followed by continual cell culturing without drugs for 48 h exhibited potent cytotoxicity, suggesting that the sigma-2 ligand delivered a significant amount of SMC into cells in 2 h.

Several lines of evidence suggest that **SW-III-123** or **SW-IV-134** entry to cancer cells may be mediated by sigma-2 receptors [41, 43, 44]. (1) **SW-III-123** and **SW-IV-134** have a high affinity for σ_2 receptors (Fig. 5.4) and can block the uptake of the fluorescent probe **SW120** into cells. (2) The sensitivity of breast cancer cells to **SW-IV-134** appears to correlate with σ_2 receptor densities of the cells. (3) The cytotoxicity of **SW-IV-134** can be partially blocked by the σ_2 receptor antagonist, **RHM-1**.

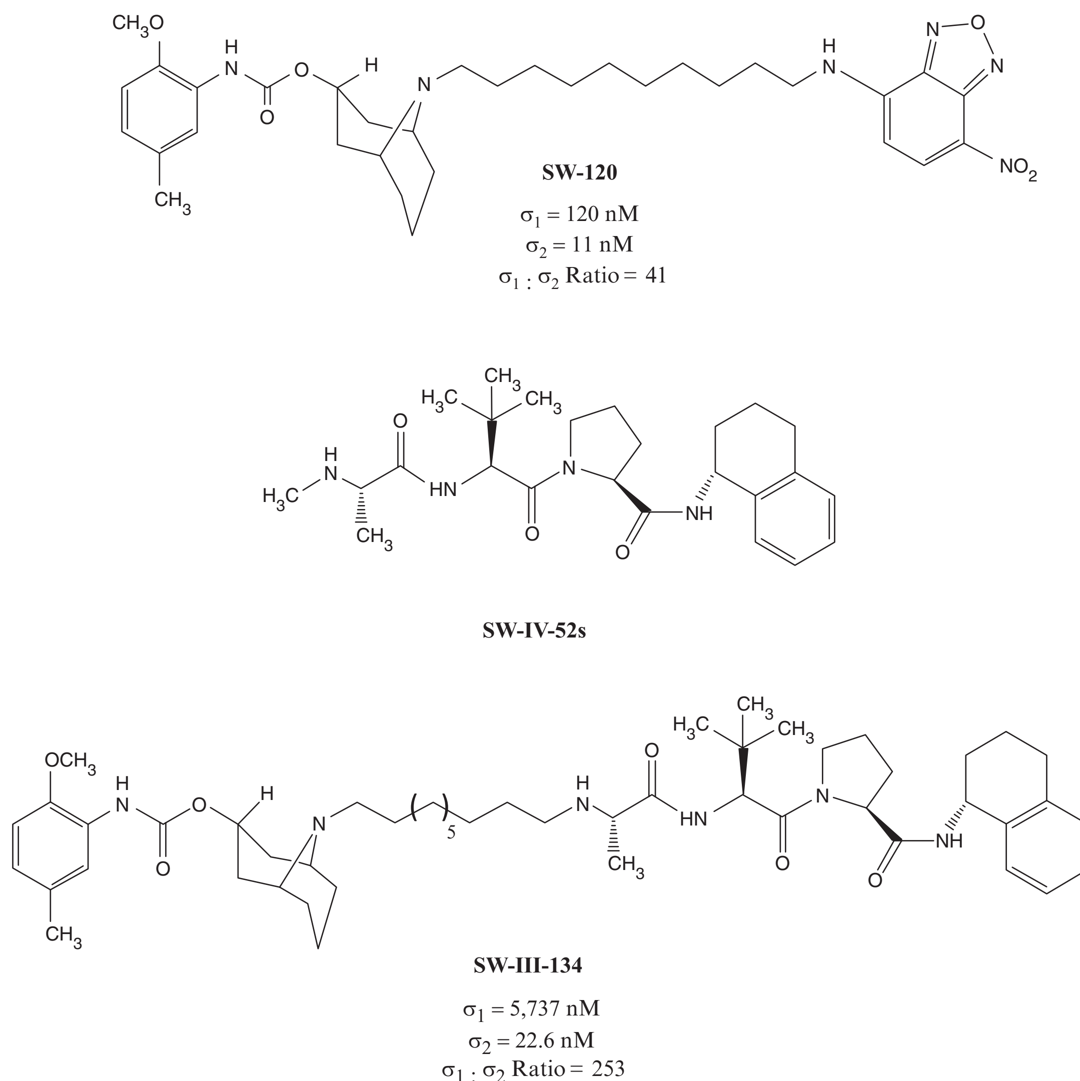

Fig. 5.4 Structure of the fluorescent probe **SW120** and the sigma-SMAC analog, **SW-III-134**

In conclusion, σ_2 receptor ligands can effectively deliver cell death-inducing peptides or peptide mimetics into cancer cells. σ_2 ligand-conjugated peptides exemplify a novel class of therapeutic drugs for treating various cancers.

5.2.4 Delivery of an Alpha-Emitting Radionuclide

Astatine-211 is a radioactive element with a half-life of 7.21 h that decays through the emission of a high-energy alpha particle. Alpha-particles induce cell death by causing DNA damage. Cells only within the immediate proximity to the radioactive decay event are affected. Makvandi et al. reported that replacement of the iodo group of **RHM-4** with astatine-211 ([^{211}At]**MM3**) [45] (Fig. 5.5) resulted in a radiolabeled compound having picomolar affinity for the σ_2 receptor in in vitro binding assays using the breast cancer cells, MDA-MB231 and EMT6 cells. Biodistribution studies in a mouse xenograft model of breast cancer showed a maximal tumor

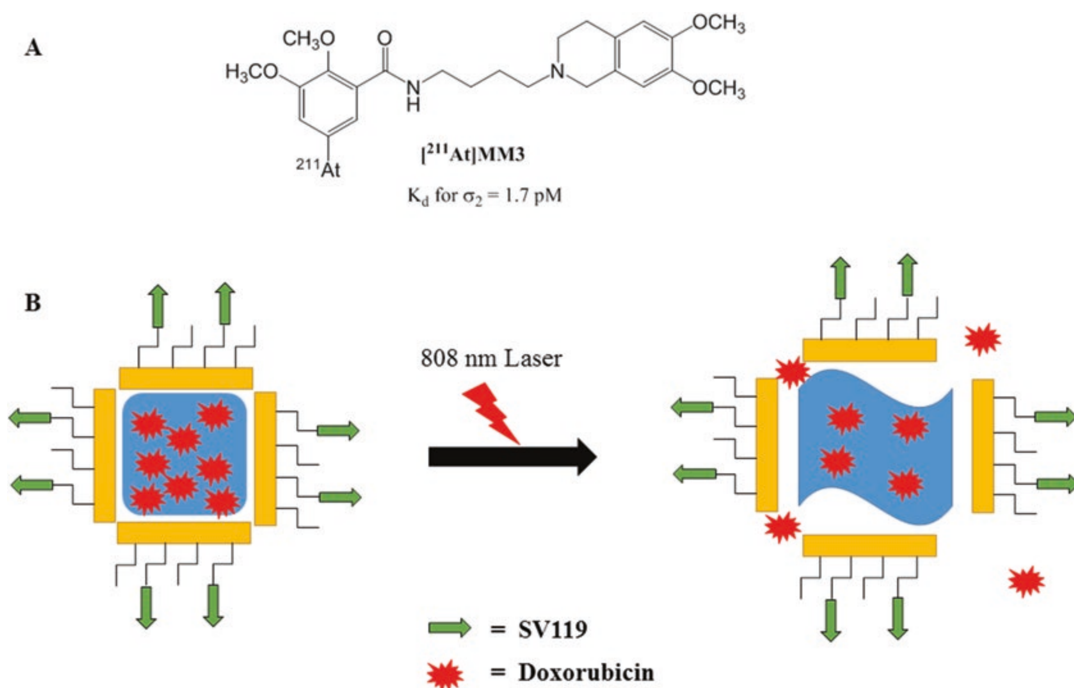

Fig. 5.5 Structures of [^{211}At]**MM3**, a target alpha emitter (**a**) and the mechanism of release of doxorubicin from **SV119**-pegylated gold nanocage (**b**)

to muscle ratio of 9.02. Estimated human dosimetry was below organ radiation limits. Therefore, [^{211}At]MM3 represents an excellent example of a σ_2 receptor ligand to serve as a receptor-targeted alpha radiotherapeutic for the treatment of breast and other cancers.

5.2.5 Delivery of Nanoparticles Loaded with Cancer Therapeutic Drugs

Gold nanocages (AuNCs) are a novel class of contrast enhancement and photothermal agents for cancer detection and treatment. AuNCs undergo a phototheral reaction and kill surrounding cancer cells by hyperthermia. The interior of the AuNCs can also be loaded with an anticancer drug for selective delivery of the agent to cancer cells, which can be released in a controllable fashion. The efficacy of drug-loaded AuNCs largely depends on the concentration of the AuNCs delivered to the tumor. Functionalization of the surface of AuNCs with tumor targeting ligands is an effective strategy for accomplishing this goal. Xia et al. conjugated **SV119** to poly (ethylene glycol) functionalized AuNCs to produce **SV119**-PEG-AuNCs [46, 47] (Fig. 5.5). **SV119**-PEG-AuNCs can block the uptake of **SW120** in MDA-MB435 cells, suggesting that **SV119**-PEG-AuNCs have a good affinity for σ_2 receptors**. SV119**-PEG-AuNCs exhibited significantly increased cellular uptake compared to AuNCs alone, also suggesting that **SV119** successfully delivered AuNCs into cells. The interior of AuNCs were also loaded with the anticancer drug doxorubicin (DOX), and then the AuNCs/DOX were conjugated with **SV119**-PEG to produce **SV119**-PEG-AuNC/DOX. The data showed that **SV119-**PEG-AuNC/DOX were internalized by MDA-MB435 cancer stem cells. The DOX was released from the AuNCs by irradiating the cell culture with a diode laser centered at 808 nm (Fig. 5.5). This treatment resulted in the significant reduction in self-renewal capability of MDA-MB435 cancer stem cells. These results

demonstrate that the **SV119-**AuNCs conjugate can serve as a new platform for simultaneous photothermal chemotherapy of solid tumors and cancer stem cells.

Li et al. has reported the covalent linking of **SV119** with polyethylene glycol-dioleyl amido aspartic acid conjugate (PEG-DOA) to generate a novel functional lipid, **SV119**-PEG-DOA [48]. This approach was used for the preparation of targeted liposomes to enhance their uptake in cancer cells by σ_2 receptor-mediated internalization. In addition, doxorubicin (DOX) was loaded into the liposomes. The **SV119**-functionalized liposomes exhibited significantly increased cellular uptake by DU-145, PC-3, A549, 201T, and MCF-7 tumor cells. Furthermore, the DOX-loaded **SV119** liposomes showed significantly higher cytotoxicity to DU-145 cells compared to the DOX-loaded unmodified liposomes. The results suggest that **SV119-**modified liposomes might be a promising drug carrier for the delivery of targeted liposomes to solid tumors.

5.3 σ_2 Receptor Ligands as Potential Sensitizers of Cancer Therapeutics

Bowen et al. were the first to demonstrate that a σ_2 receptor ligand, CB-184, potentiated the cytotoxicity of doxorubicin both in drug-sensitive (MCF-7) and drug-resistant (MCF-7/Adr-) cell lines [19]. Hawkins et al. later demonstrated that σ_2 receptor ligands potentiate conventional chemotherapeutics both in vitro and in vivo [28, 49]. Combinations of **SV119** with gemcitabine or paclitaxel demonstrated increased apoptosis compared to **SV119**, gemcitabine or paclitaxel alone in mouse (Panc-02) and human (CFPAC-1, Panc-1, AsPC-1) pancreatic cancer cell lines. Mice bearing tumor allografts were treated with **SV119** (1 mg/day) in combination with paclitaxel (300 microg/day) over 7 days; the combination treatment of **SV119** and paclitaxel increased mouse survival compared to either **SV119** or paclitaxel treatment alone. Treatment of **SV119** on alternating days (1 mg/day) in combination with weekly treatment of gemcitabine (1.5 mg/week) for 2 weeks also showed a significant survival benefit. No gross toxicity was noted in serum biochemistry data or on necropsy. In addition, **SW43** in combination with gemcitabine exhibited increased apoptosis and decreased viability over **SW43** or gemcitabine alone in pancreatic cancer cells under cell culture conditions. The in vivo model showed that the combination treatment with **SW43** and gemcitabine resulted in greater reduction in tumor volume than either **SW43** or gemcitabine treatment alone. These results highlight the potential utility of the σ_2 receptor agonists as an adjuvant treatment in pancreatic cancer.

5.4 σ_2 Receptor Ligands as Potential Drugs for Neurological Diseases

By definition, the σ_2 receptor is the binding site of a unique panel of drugs including psychotomimetic opioids and neuroleptic drugs [18]. Some σ_2 receptor ligands have been shown to have neurological effects [50]. Izzo and colleagues reported the discovery and development of novel σ_2 receptor antagonists as therapeutics targeting beta amyloid (Aβ) 1–42 oligomers for treating Alzheimer's disease (AD) [51, 52]. Soluble oligomers of Aβ have been recognized to be early and key intermediates in AD-related synaptic dysfunction. Soluble Aβ oligomers are thought to cause synaptic dysfunction and loss and impair rodent memory. Alterations in membrane trafficking induced by Aβ oligomers are believed to mediate this synaptic dysfunction. By screening a library of central nervous system drug-like small molecules for their abilities to reverse Aβ-induced membrane trafficking deficit in primary neurons, active compounds (i.e., hit compounds) were identified. In order to identify the molecular target of the active hits, these compounds were examined for their activities for 100 receptors and enzymes in the central nervous system. As a result, these compounds were found to have a high affinity for only the σ_2 receptor. These ligands displaced Aβ oligomer binding to synaptic puncta, and prevented and reversed Aβ

oligomer-induced synapse loss in primary neuronal cultures, as well as reversed memory loss in transgenic mouse models of AD. These σ_2 receptor ligands, which we found to be antagonists in functional assays, may represent a novel class of drugs for treating AD patients.

5.5 σ_2 Receptor Ligands Could be Potential Drugs for Inflammatory and Autoimmune Diseases

Fresno et al. [53] reported that σ_2 agonists attenuated T lymphocyte activation. The σ_2 agonists **BD-737** and **CB-184** decreased the induction of protein and mRNA expression of Interleukin (IL)-2, tumor necrosis factor (TNF)-α, and cyclooxygenase (COX)-2 by activated Jurkat T cells. BD-737 and CB-184 inhibited the induced transcriptional activity of nuclear factor (NF)-κB or nuclear factor of activated T cells (NFAT). These results showed that σ_2 agonists exhibited anti-inflammatory actions through the inhibition of NFAT-dependent transcription. The data suggest that σ_2 agonists could be potential drugs for treating inflammatory and autoimmune diseases. Further studies are needed to demonstrate the anti-inflammatory effects of σ_2 receptor ligands in animal models of inflammation.

5.6 Summary and Outlook

The σ_2 receptor continues to be an important protein in the field of cell biology. The high density of this receptor in proliferating versus quiescent breast tumors indicates that the σ_2 receptor is an important biomarker for measuring cell proliferation, in particular the ratio of proliferating to quiescent cells (i.e., the proliferative status) of a tumor. Since σ_2 receptor agonists can kill tumors by both apoptotic and non-apoptotic pathways, it also suggests that this receptor is a potential target for the development of cancer chemotherapeutic agents. σ_2 agonists can also function as chemosensitizers of known cancer chemotherapeutics and have the potential to deliver cancer therapeutics to tumor cells either through direct conjugation or via the functionalization of gold nanocages or liposomes. The recent observation that σ_2 antagonists can block Aβ1–42 oligomer synaptic dysfunction and improve performance in memory tasks in transgenic models of AD indicate a new potential therapeutic target in treating neurodegeneration. The next step in the evolution of research on the σ_2 receptor is the complete molecular characterization of this protein, including the amino acid sequence and molecular weight of the primary protein sequence, and the gene responsible for encoding this protein. The clinical translation of promising σ_2 agonists and antagonists in the treatment of cancer and Alzheimer's disease also represents an important step in the evolution of this protein from an obscure and poorly understood radioligand binding site to an important therapeutic target in the treatment of a variety of pathological conditions.

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Sigma 1 Receptor and Ion Channel Dynamics in Cancer

6

Olivier Soriani and Raphaël Rapetti-Mauss

Abstract

SigmaR1 is a multitasking chaperone protein which has mainly been studied in CNS physiological and pathophysiological processes such as pain, memory, neurodegenerative diseases (amyotrophic lateral sclerosis, Parkinson's and Alzheimer's diseases, retinal neurodegeneration), stroke and addiction. Strikingly, G-protein and ion channels are the main client protein fami lies of this atypical chaperone and the recent advances that have been performed for the last 10 years demonstrate that SigmaR1 is principally activated following tissue injury and disease development to promote cell survival. In this chapter, we synthesize the data enhancing our comprehension of the interaction between SigmaR1 and ion channels and the unexpected consequences of such functional coupling in cancer development. We also describe a model in which the pro-survival functions of SigmaR1 observed in CNS pathologies are hijacked by cancer cells to shape their electrical signature and behavior in response to the tumor microenvironment.

Keywords

Sigma1R • Ion channels • Cancer • Cell cycle • Tumour microenvironment • Extra cellular matrix • Integrin • Angiogenesis • Invasion

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6.1 Introduction

The sigma 1 receptor (SigmaR1) is a ubiquitous chaperone protein mainly expressed in the brain and the liver. Within the cell, this 25-kDa protein is located at the endoplasmic reticulum (ER)-mitochondria interface (mitochondria ER-associated membrane, MAM) but can be dynamically redistributed to the plasma membrane (PM) and the nucleus envelope [1, 2]. The chaperoning function of Sig1R emerged 10 years ago in a study showing that ER stress activates SigmaR1 which binds with client proteins such as IP3 receptors (IP3-R) to preserve Ca^{2+} homeostasis and cell survival [3]. Ever since, cumulative studies have been demonstrating that SigmaR1 directly interacts with membrane proteins such as ion channels and G protein-coupled receptors to shape neuronal activity and enhance cell survival [1, 2, 4]. The function of this fascinating protein has been extensively explored in the brain and a growing body of evidence indicates that SigmaR1 is activated upon tissues injury and is therefore associated with many neurophysiopathological contexts. Among them, stroke [5], neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) [6–7], Alzheimer's disease (AD), retinal degeneration [8, 9], pain and cocaine addiction [10] are specifically scrutinized. Given this context, SigmaR1 is now accepted as a multitasking chaperone functioning as a pluripotent modulator in living systems (Fig. 6.1).

In another hand, the question of the putative involvement of SigmaR1 in cancers remains poorly documented. It should be noticed that many groups have pointed out the possible involvement of Sigma receptors in cancer cell physiology since the early 90's. The arguments

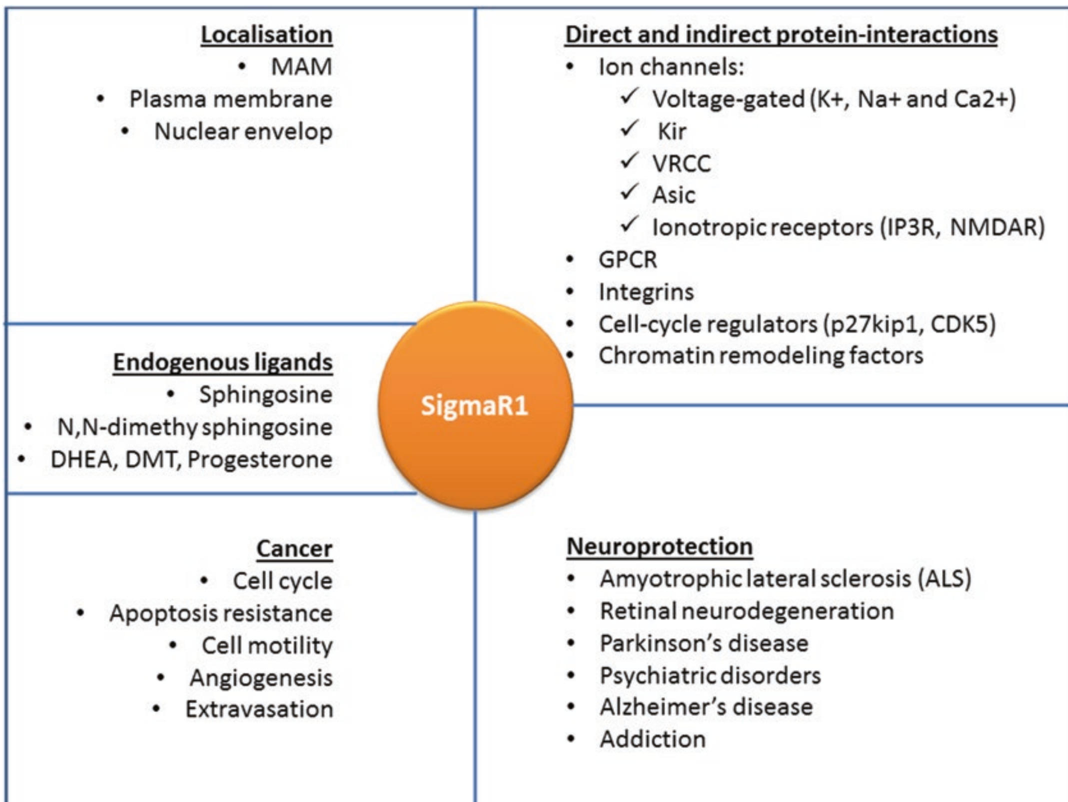

Fig. 6.1 The multitasking sigma-1 receptor chaperone. Overview of some sigma-1 receptor molecular interactions and potential therapeutic applications (Adapted from Refs. [1, 10–15])

were supported by the high density of sigma receptor binding sites in cancer cell lines, and the anti-proliferative or cytotoxic effects produced by exogenous sigma ligands on cancer cell lines *in vitro* [16, 17]. Despite the number of descriptive studies published on this subject, the innate function of the protein in cancers and the associated molecular mechanisms have long remained unanswered questions.

Ion channels represent the first class of therapeutic targets after the G-protein coupled receptors family [18]. Expressed in all cell types, they participate in many functions that range from cell signaling to the regulation of ion and water homeostasis. During the past 20 years, accumulating evidence has pointed out the role of ion channels in cancer. The synthesis of the work, which has been conducted in this domain now strongly suggests that the electrical signature of cancer cells is remodeled by the aberrant expression of channels, often absent from the healthy tissue, which in turn contributes to hallmarks of cancer [19–25]. As a consequence, ion channels are now considered as an exciting class of protein to provide new candidates for prognosis, diagnostic, but also therapeutic targets to counteract disease progression. For the latter, one of the main drawbacks is that cancer cells express the same ion channels as those expressed in healthy tissues such as heart or brain, rendering the therapeutic use of specific blockers such as ion channel toxins hazardous. As a consequence, understanding the specific modalities of ion channel function and regulation in cancer tissues remains a central question.

In this chapter, we will focus on mechanisms linking SigmaR1 to ion channels and on the role of [SigmaR1:Ion channel] complexes in cancer development, two domains in which our group has contributed to for 10 years.

6.2 Sigma Receptors and Ion Channels

6.2.1 Early Studies

Among the client proteins of the SigmaR1, the family of ion channel remains the one for which the modalities of interaction with the chaperone at both functional and molecular levels is the best described so far (for reviews see [1, 4, 26]). The first clues linking sigma receptors to ion channels arose from early pharmacological studies long before the molecular nature of SigmaR1 was revealed by Hanner in 1996 [27]. For example, it was observed that so-called sigma binding sites were closely associated to NMDA receptors and that sigma ligands could modulate NMDA receptor activity [28–31]. It was also reported that voltage-dependent and leak K^+ currents were blocked by several sigma ligands, inducing cell depolarization in neurons of the hypogastric ganglion and glioblastoma cell lines [32, 33]. Our group contributed to the first mechanistic insights in a series of electrophysiological studies conducted in primary frog pituitary cells. We demonstrated that acute application of several sigma ligands such as (+)pentazocine, DTG and igmesine provoked a depolarization and a stimulation of action potential firing. These effects were attributable to the modulation of biophysical parameters of several voltage-dependent K^+ channels (i.e. the delayed-rectifier, the transient outward and the M-current) but also a tonic outward K^+ channel [34–36]. Depending on current subtype, we observed that sigma ligands could either affect current density, voltage- and time-dependent inactivation or deactivation kinetics. Interestingly, inhibition or tonic activation of G-protein function with either cholera toxin, GTPγS or GDPβS, deeply altered the effects of sigma ligands on K^+ currents and global electrical activity. On the other hand, similar electrophysiological studies performed in rat pituitary cells indicated that sigma ligands could also inhibit K^+ channels in a G-protein independent manner [37]. This apparent discrepancy may now be explained by evidence that SigmaR1 can participate in the coupling of an ion channel to a membrane receptor, including a G-protein receptor [11, 38, 39]. Altogether, these reports based on the use of exogenous sigma ligands suggested the existence of a functional, but complex, link between SigmaR1, K^+ channels and eventually G-protein-coupled receptors, depending on the

ion channel subtype as well as the model being explored (Table 6.1).

6.2.2 SigmaR1 as a Ion Channel Chaperone

A better understanding of the molecular mechanisms involved as well as the innate function of SigmaR1 arose from a series of studies manipulating SigmaR1 expression. In experiments carried out in *Xenopus Laevis* oocytes, co-expression of sigmaR1 and voltage-dependent ion channels mRNA showed that SigmaR1 was able to reduce current amplitude and accelerate current inactivation in the absence of sigma ligands [40]. In HEK293 cells, we further demonstrated that over expression of SigmaR1 was sufficient to delay activation rate of a volume-regulated Cl^- channel (VRCC) involved in cell volume regulation [12]. Previous models were generally based on a mechanism of action similar to that of a classical receptor, in which an endogenous or exogenous agonist is generally required to trigger the function. The results showing that the presence of SigmaR1 was sufficient to modulate ion channel function per se shed new light on the understanding of SigmaR1 modality of action, considering the protein as a constitutive regulator whose activity could be modulated by ligands. Consistent with this new point of view, Hayashi and Su unveiled in 2007 the ion channel-chaperoning function of SigmaR1 based on ER-stress triggered association between SigmaR1 and IP3-R at the level of mitochondria-associated membrane (MAM) [3]. Moreover, several groups suggested on the basis of co-immunoprecipitation experiments, the existence of proximity interaction between SigmaR1 and PM ion channels from different families (including voltage activated K^+, Ca^{2+} and Na^+ channels but also acid-sensing ion channels (ASIC) [13, 26, 40–43] (Table 6.1). The studies we performed with human-ether-à-gogo-related-gene (hERG or Kv11.1 or KCNH2) and Nav1.5 channels exemplify the insights that have been obtained during the last years.

6.2.3 The Example of SigmaR1:hERG Interaction

hERG is a voltage-gated K^+ channel mainly involved in cardiac, endocrine and neuronal cell excitability [44, 45]. As a member of the Kv family, the functional channel is formed by the association of 4 α-subunits, each of which is composed of 6 transmembrane domains [46]. Co-expression of SigmaR1 and hERG mRNA in *Xenopus* oocytes resulted in a dramatic increase in current density without modifying voltage-dependency or kinetic parameters. Using a cell line constitutively expressing hERG (K562), we observed that the silencing of endogenous SigmaR1 by shRNA inhibited current amplitude. Interestingly, detection of hERG expression at the plasma membrane by flow cytometry in non-permeabilized cells revealed that current reduction observed in SigmaR1-silenced cells could be attributed to the reduction of channel density at the cell surface. Two main forms of hERG channels can be observed in western blot, corresponding to glycosylation maturation process in mammalian cells: a light, immature form corresponding to the ER-associated immature pool, and a heavier, fully glycosylated mature form corresponding to the functional fraction expressed at the plasma membrane [41, 47]. We observed that in SigmaR1-silenced cells, the maturation process was altered since the ratio between steady-state contents of mature and immature forms was reduced. Pulse-chase experiments revealed that SigmaR1 expression not only enhanced channel maturation but also increased α-subunit stability at the PM [41]. Not surprisingly, co-immunoprecipatation experiments suggested a close association between SigmaR1 and both mature and immature forms of hERG subunits, further indicating that SigmaR1 chaperones hERG trafficking process. Accordingly, it was found that SigmaR1 is associated with COP-I vesicles which are involved in complex functions in intra-Golgi trafficking [48, 49]. Further studies are necessary to determine how COP-I dependent trafficking affects hERG plasmalemmal expression.

Table 6.1 Examples of interactions between SigmaR1 and ion channels

	Effect of sigma agonists	Constitutive regulation	Proximity interaction	G protein fonctional interaction	References
IK_{DR}/Kv1.5	↓current density	↓current density	Yes	Yes (frog pitutary)/No (Xenopus oocyte)	[36, 40]
I_A/Kv1.4	↑steady-state inactivation	↑inactivation	Yes	Yes (frog pitutary)/No (Xenopus oocyte)	[35, 40]
	↑inactivation rate				
	↓current density				
Kv2.1	↓current density	nd	nd	Yes	[39]
I_M	↓steay-state activation	nd	nd	nd	[34]
	↑de-activation				
hERG	↓current density	↑trafficking	Yes	no	[50, 86]
Kv1.2	↑channel trafficking	none	Yes	nd	[52]
	↑current density				
Kv1.3	↓current density	↓inactivation rate	Yes	no	[15, 84]
Kir2.1	↓current density	none	Yes	nd	[14]
Nav1.5	↓current density	↓current density	Yes	nd	[43, 87, 88]
	↑steady-state inactivation				
	↓SigmaR1:Nav1.5				
L-type voltage-gated Ca^{2+} channel	↓current density	nd	Yes	nd	[89]
Asic1a	↓current density	nd	Yes	nd	[43, 90]
NMDA R	↑current density	none	Yes	nd	[51, 53]
	↑channel trafficking				
	↑SigmaR1:NMDAR binding				
IP3R (ER)	↑Ca^{2+} release	↓Ca^{2+} release	Yes (through Ank220)	Yes	[42, 91]
	↓[SigmaR1:Ank220]:IP3R binding				
IP3R (MAM)	↑Ca^{2+} release	none	Yes	nd	[3]
	↑channel stability				
	↑SigmaR1:IP3R binding				
ORAI1	↓Ca^{2+} influx	↓Ca^{2+} influx	Yes (through STIM1)	nd	[55]
	↓Stim1:ORAI1 binding	↓Stim1:ORAI1 binding			
VRCC	↓current density	↓activation rate	n.d.	no	[12]

nd not determined

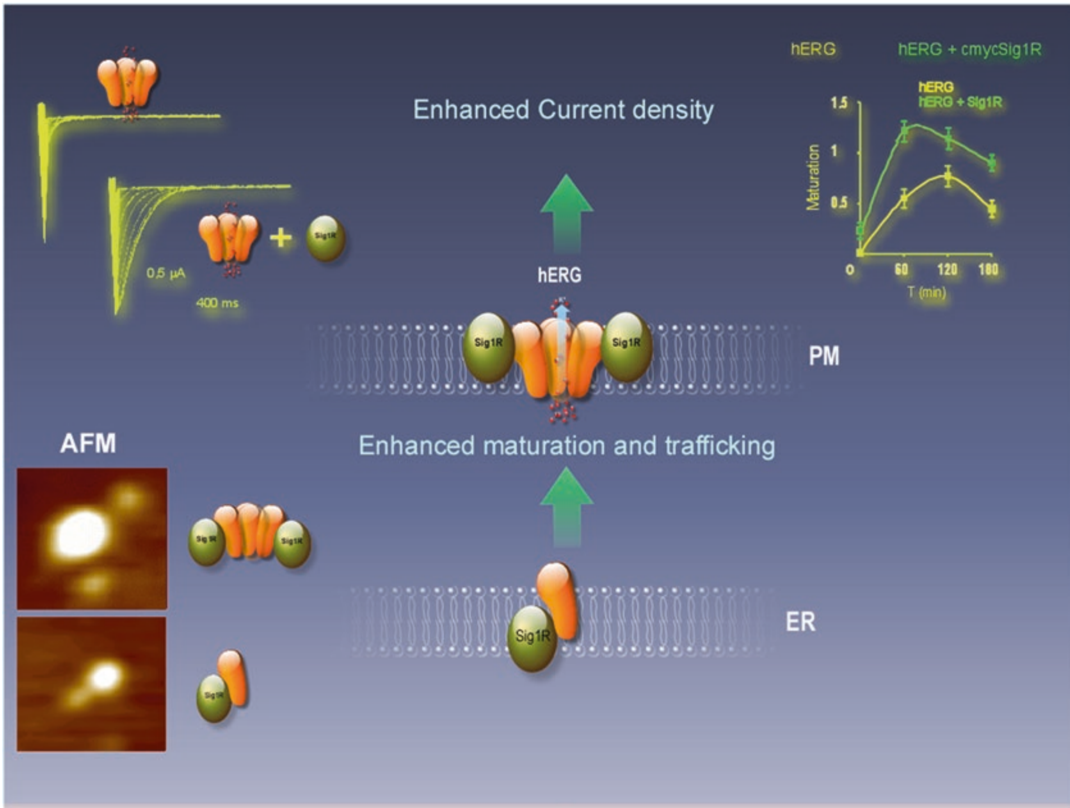

Fig. 6.2 hERG channel regulation by the SigmaR1 chaperone. SigmaR1 binds α-subunit of the channels and stimulates maturation, trafficking and stability at the PM leading to increased current density. Atomic force microscopy reveals that each of the 4 subunit of the channel can bind to 1 SigmaR1 [41, 50]

To delve into the intimacy of the interaction between the two proteins, we performed atomic force microscopy imaging and revealed that the distance between the partners was consistent with a direct protein:protein interaction. Moreover, hERG dimers and tetramers became both singly and doubly decorated by sigma-1 receptors while hERG monomers were only singly decorated. The angles between pairs of sigma-1 receptors bound to hERG tetramers were found at ~90 and ~180° in a ratio of ~2:1, indicating that the sigma-1 receptor interacts with hERG with 4-fold symmetry [50]. Time-resolved Förster resonance energy transfer (HTR-FRET) experiments performed in intact cells revealed that the SigmaR1:hERG complexes could be detected at the PM. Based on these results, we proposed a model in which SigmaR1 co-assembles to channel α-subunit at the level of the ER and participates in channel trafficking by stimulating protein maturation and increasing ion channel expression and stability at the plasma membrane (Fig. 6.2).

The protein:protein interaction between SigmaR1 and pore-forming channel subunits was confirmed for other ion channels including Nav1.5, ASIC1, the NMDA receptor mGLUN1 and Kv1.2 channels in recent reports combining AFM imagery and functional approaches [13, 43, 51, 52]. In the breast cancer cell line MDA-MB-231, similar to the results obtained with hERG channel, we found that SigmaR1 binds to the cardiac Nav1.5 channel (SCNA5) with a 4-fold symmetry, and increases Na^+ current density [43].

In rat brain, SigmaR1 ligands such as (+)pentazocine or cocaine stimulate NMDA receptor and Kv1.2 channel trafficking to the PM, revealing

one of the probable molecular mechanisms underlying SigmaR1-associated memory process or cocaine addiction, respectively [52, 53]. Remarkably, the chaperoning of membrane ion channels by SigmaR1 can be extended to intracellular channels since ER-stress and sigma agonists trigger SigmaR1 binding to IP3 receptors in MAM to protect IP3R from degradation [3]. Altogether, these results are in line with a model in which SigmaR1 behaves as a chaperone regulating ion channel trafficking in various models as described in Fig. 6.2 and Table 6.1.

6.2.4 ER:PM Transregulation of Ion Channels by SigmaR1

Alternative modalities of interactions between SigmaR1 and ion channels have been suggested. In particular, examples indicating that ER pools of SigmaR1 regulate ion channel located at PM at ER-PM junctions were recently reported. One of them concerns the process of store-operated calcium influx. The emptying of ER Ca^{2+} stores induces the binding of the STIM1, a regulatory protein localized in the ER membrane, to the plasmalemmal Ca^{2+} channel ORAI1. The binding of STIM1 to ORAI1 provokes the opening of the channels and Ca^{2+} influx from the outside of the cell to the cytoplasm to restore ER Ca^{2+} pools [54]. Srivats and collaborators recently showed that STIM1 is associated to SigmaR1 in the ER membrane. The presence of SigmaR1 slowed the recruitment of STIM1 to ER-PM junctions and reduced binding of STIM1 to PM Orai1, in turn inhibiting store-operated calcium influx [55]. A second report proposed that SigmaR1 located at the ER could negatively regulate kinetic parameters of Kir2.1 K^+ channels expressed at the plasma membrane of motoneurons [14]. These hypothesis are supported by electron microscopy studies showing that ER pools of SigmaR1 are localized at the ER-PM junction in motoneurons, ganglion cells and dorsal root ganglion neurons [56, 57].

Given that SigmaR1 is activated under ER-stress conditions, it emerges from these findings that the remodeling of cell electrical signature and/or Ca^{2+} homeostasis may be one of its general functions [26, 58]. Accordingly a number of studies have associated, in an ion channel-dependent manner, SigmaR1 to brain physiologic and physiopathologic processes such as pain, memory neurodegenerative diseases and cocaine addiction (for reviews: (1, 2, 51)). In the next part, we will focus on the significance of SigmaR1:ion channel interaction in cancer.

6.3 Regulation of Ion Channels in Cancer

During the past decade, aberrant expression of ion channels in cancer tissues has been described and clearly associated to the acquisition of malignant phenotype and metastasis progression. Involved in all the hallmarks of cancer, their contributions range from the control of cell proliferation to the regulation of invasiveness and metastatic spreading (for reviews on this emerging topic please see: [20, 22, 25, 59, 60]. Ion channels might represent a promising class of therapeutic targets but major drawbacks exist since toxins would inhibit channels expressed both in tumor cells and healthy tissues such as heart and CNS. Understanding the molecular pathways involved in the regulation of ion channels in tumor cells is therefore a prerequisite to specifically target their functions in cancer tissues. In particular, ion channel regulating protein such as auxiliary β-subunit has recently been under scrutiny by several groups [61, 62]. Given the functional relationship between SigmaR1 and ion channels, revealed mainly in CNS models, we chose to explore the putative role of SigmaR1 and associated ion channels in cancer cell behavior.

6.3.1 Role of SigmaR1 in Cell Growth Regulation

Early studies by Bowen's group described that incubation of various cancer cell lines with sigma ligands inhibited cell growth in vitro. Interestingly, the arrest of cell growth observed was accompa-

nied by a cell swelling [17]. This early observation was compelling since volume regulation is a physiological feature involved in cell cycle and apoptosis, two processes regulating tumor cell growth [63].

6.3.2 SigmaR1 Regulates Regulatory Volume Decrease (RVD) During G1/S Transition

Cell cycle progression depends on an increase in cell volume, and the capacity for regulatory volume decrease (RVD) changes during the cell cycle. Accordingly, cell volume culminates in the M phase and is the smallest in the G1 phase and was found to be increased in parallel to the G1-S transition [63, 64]. The direct effects of changes in cell volume on the cell cycle control are still not clear, but it seems that cell swelling induced by hyposmotic stress in general stimulates signaling pathways involved in cell cycle progression (for review see [63]). In particular, KCl-dependent osmotic water efflux allows cells to control cell volume in the course G1/S transition, this mechanism being the consequence of the activation of K^+ and Cl^- channels (VRCC) in response to hypotonic shock. In support of this, pharmacological blocking of K^+ and Cl^- conductances involved in RVD inhibits cell cycle in the G1 phase [65, 66]. We observed in small cell lung carcinoma (SCLC: NCI-H209 and NCI-H146) and T leukemia (Jurkat) cell lines that sigma ligands including igmesine, (+)pentazocine and DTG were able to abrogate RVD by inhibiting both voltage-dependent K^+ channels (Kv) and VRCC. Remarkably, these effects were accompanied by the inhibition of cell growth. $p27^{kip1}$ and p21Cip1 are inhibitors of cyclin D:CDK complexes and cyclin E 4/6 CDK-2, both responsible for the phosphorylation of pRb. PRb phosphorylation is a critical step in the G1 / S transition because it leads to the release of transcription factors required for the S-phase of activation of genes such as cyclin A [67]. In cells deficient in pRb, and it is the case of SCLC, $p27^{kip1}$ or $p21^{Cip1}$ are still able to inhibit the cyclin E-CDK-2, which in this context, directly controls the activation of cyclin A [68]. We found that cell incubation with either sigma ligands or Kv (TEA or 4-AP) and VRCC (NPPB) channel blockers, resulted in $p27^{kip1}$ accumulation and decrease in expression of cyclin A. These results are in line with a cell cycle arrest in G1 of the cell cycle. Moreover, it is interesting to note that the level of expression of $p21^{Cip1}$was not affected by incubation with sigma ligands, highlighting the specificity of sigma receptor-dependent regulation. These early results suggested that SigmaR1 was functionally associated with the control of cell cycle by regulating ion channels involved in RVD and that modulation of this coupling by sigma ligands could stop cell division at the end of the G1 phase (Fig. 6.3, left).

6.3.3 SigmaR1 Reduces Apoptosis by Regulating AVD

Cell shrinkage or AVD is hallmark of apoptosis and has been characterized as an early event required for apoptosis triggering [69], and accumulating evidence indicates that preventing cell volume regulation after shrinkage is associated with triggering of apoptosis. AVD results from a loss of KCl via K^+ and Cl^- channels, and concomitant loss of water [63, 69, 70]. We found that overexpression of SigmaR1 in HEK293 cells did not change the amplitude of VRCC in response to a hypo-osmotic shock. However, the analysis of current kinetic properties unveiled that SigmaR1 slows its activation down following hypo-osmotic stress. Consistent with this result, the RVD is delayed in HEK293 cells overexpressing the SigmaR1. It is important to note that since SigmaR1 does not inhibit the Cl^- channel, RVD is not prevented when SigmaR1 is overexpressed, therefore the cell cycle is not altered. Importantly, we found that SigmaR1 significantly reduced staurosporine-induced AVD, suggesting that the presence of SigmaR1 contributes to cancer cell apoptosis resistance [12] (Fig. 6.3, right). This idea is coherent with other reports describing the pro-survival role of SigmaR1 in neurodegenerative diseases [9, 71, 72]. Remarkably, the initial work of Hayashi and Su demonstrating

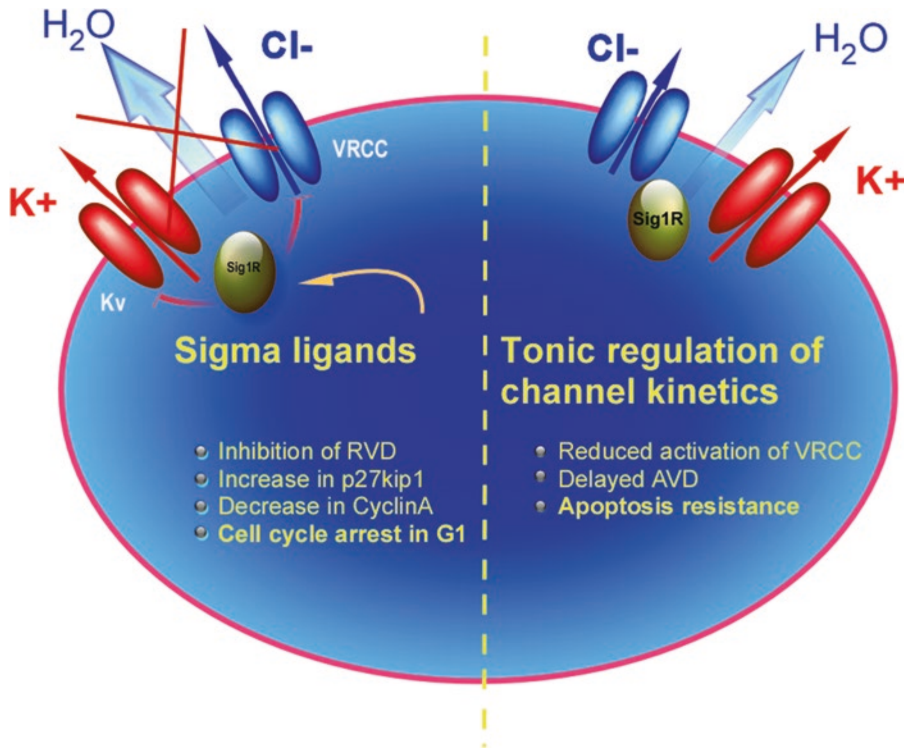

Fig. 6.3 SigmaR1 regulates cell cycle and apoptosis in cancer cells. *Left*, cancer cell incubation with sigma ligands inhibits cell cycle at the end of the G1 phase. This process is a consequence of the inhibition of both VRCC and Kv channels, abrogating RVD. *Right*, Overexpression of SigmaR1 in tumor cells sufficiently alters the properties of the Cl- channel (and possibly K+ channels) to restrict the amplitude of the AVD in response to pro-apoptotic signals, increasing cell resistance to apoptosis [12, 15]

the chaperoning activity of SigmaR1 in 2007, described a pro-survival activity of SigmaR1 involving its interaction with a Ca^{2+} channel, i.e. the IP3 receptor [3]. Accordingly, it can be proposed that the SigmaR1-dependent shaping of cell electrical and Ca^{2+} signatures participates to cell survival in injured tissues occurring in pathological contexts such as ALS or stroke [5, 58, 72]. Since SigmaR1 is over expressed in many cancer cells, this raises a possibility in which the pro-survival function of SigmaR1 is hijacked by cancer cells to promote their own survival in metabolic-restricted conditions encountered in cancer tissue. We further explored this idea by addressing the function of SigmaR1 in the crosstalk between cancer cells and their microenvironment.

6.3.4 SigmaR1 Regulates the Dynamic Crosstalk Between Cancer Cells and the Extra Cellular Matrix (ECM)

The development of cancer is not limited to the accumulation of intrinsic abnormalities. Indeed, during the last 10 years, many observations have stressed the importance of interaction between pre-cancerous and cancerous cells with their microenvironment (ME) during the different stages of pathology evolution. The tumor microenvironment (TME) includes, next to cancer cells, all neighboring cells (fibroblasts, vascular cells, dendritic cells, immune cells, etc.). These cells are embedded in the extracellular matrix (ECM), which is a determinant of both normal ME and TME. By interacting with ECM

components, the tumor cell can be anchored to a particular location or on the contrary, migrate and move. The elements of the ECM are multiple and comprise macromolecules forming a hydrated gel (hyaluronic acid, proteoglycans), fibrillary proteins (collagen) and structural proteins (laminin, fibronectin, etc.) capable of interacting directly with the cells and to modulate many biological functions. At this stage, it is important to consider that tumor cells deeply restructure the ME, which in turn, greatly influences their invasive characteristics. Therefore, the characterization of the molecular actors of the crosstalk between cancer cells and TME has become a crucial objective of cancer research [19]. Ion channels expressed at the surface of tumor cells behave like microbiosensors "tasting" the chemical, physical and structural nature of the ME and producing signals that are integrated by the cell to adapt its behavior (for reviews: [21, 24, 73, 74]). hERG has been pointed out as a channel strongly involved in such mechanisms. The channels has been characterized as a biomarker of many solid tumors (colorectal cancer, glioblastoma, head and neck cancers) [75, 76] and acute or chronic leukemias [77, 78]. By forming membrane protein platforms with receptors of the TME, such as integrins (adhesion receptors of the extracellular matrix (ECM)), hERG deeply influences signaling pathways controlling in turn cancer cell spreading [79].

Taking into account the existence of interactions between SigmaR1 and ion channels involved in cancer development such as hERG, we observed, using human cancer databases (i.e. the Cancer Genome Atlas and Oncomine©), that SigmaR1 mRNA was overexpressed in CRC and myeloid leukemia (ML), two cancers for which hERG plays a crucial function [75, 78, 80]. We established a stimulation protocol of CRC or ML cells by ECM *in vitro* using a model of 3D matrix synthesized by fibroblasts (Fibroblast-derived matrix, FDM). FDM constitute a fibronectin (FN)- and collagen-rich network, which mimics the structure and the composition of in vivo mesenchymal matrices [81, 82]. We observed that cell stimulation with either FN coating or FDM evoked a rapid increase in hERG current density associated with a dramatic increase in cell motility. ECM-induced responses (FDM or FN) were abolished by functional antibodies directed against β1-integrin subunits, suggesting the involvement of a fibronectin receptor such as α5β1 integrin receptor. Interestingly, the silencing of Sigmar1R abrogated cell response to ECM, an effect that was mimicked by hERG molecular or pharmacological inhibition. At cell and molecular levels, flow cytometry-coupled FRET experiments and proximity ligation assays demonstrated that FDM induced the rapid association of hERG with the β1-integrin subunit at the PM in a SigmaR1-dependent manner. Interestingly, hERG and SigmaR1 inhibitions also inhibited VEGF secretion in a non-additive manner. Importantly, we found that SigmaR1 was required to trigger Pi3K/Akt signaling pathway following the formation of [hERG:β1-integrin] complex in response to ECM. The consequences of SigmaR1 silencing in cancer cells were also analyzed *in vivo*: using dedicated Zebrafish and mice xenograft models we revealed that the absence of SigmaR1 dramatically inhibited invasion, angiogenesis and extravasation. Altogether, these results unveiled an innate function of SigmaR1 in cancer. By shaping cancer cell electrical signature in response to ECM, SigmaR1 orchestrates the formation of channel:receptor complexes at the plasma membrane, contributing to the integration of signals from the TME and the subsequent adaptive phenotype [11] (Fig. 6.4).

6.4 Concluding Remarks and Perspectives

The comprehension of the physiological significance of SigmaR1 as well as the cellular and molecular mechanisms associated with this protein has progressed spectacularly during the past 10 years. The data accumulated describing the functional interactions between ion channels and SigmaR1 have contributed greatly to refine the picture of the contribution of SigmaR1 to diseases, and more especially to brain diseases. The

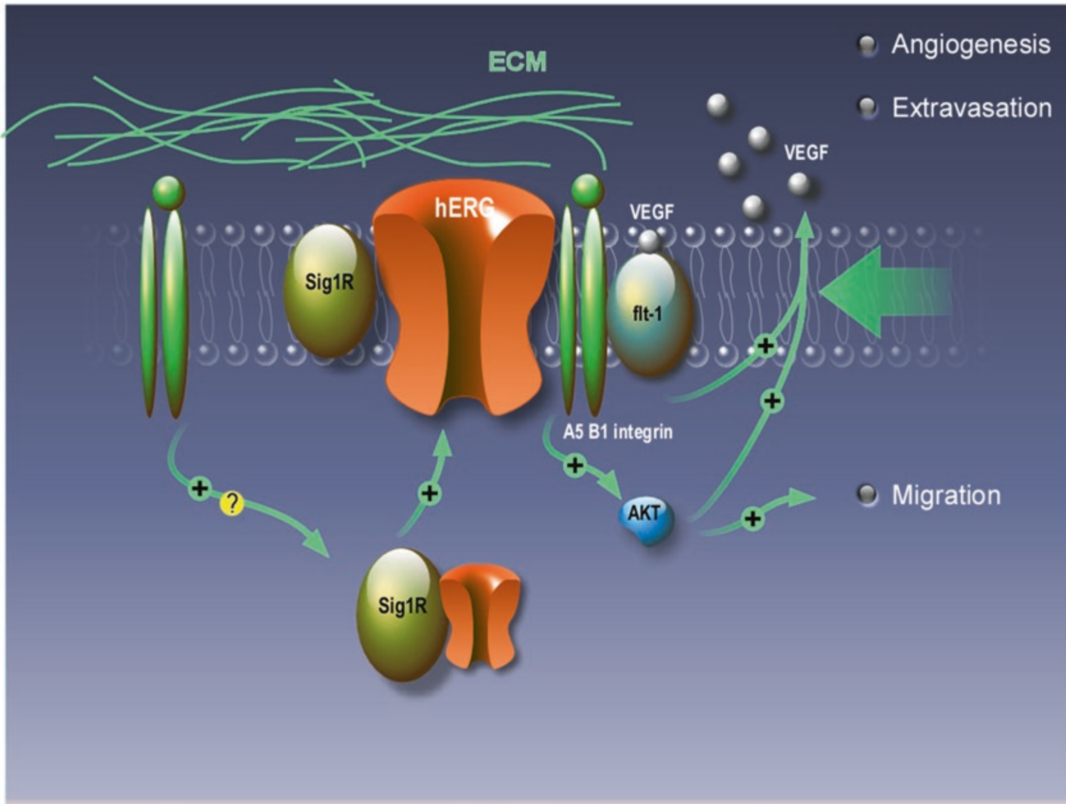

Fig. 6.4 Scheme showing a mechanistic model by which SigmaR1 triggers the formation of hERG signaling platform in response to ECM in LM and CRC. Cell contact with components of the ECM (FN) triggers a SigmaR1-dependent recruitment of hERG channels at the PM and their association to β1- integrin subunit. The protein macro-complex containing hERG, integrins, SigmaR1 and possibly Flt-1 stimulates PI3K-AKT transduction pathway, which increases migration, angiogenesis and extravasation

recent data described above demonstrate that cancers represent a new f amily of pathologies where SigmaR1:ion channel complexes may play central roles. However many questions remain to be answered.

6.4.1 What Is the Structural Basis of SigmaR1:Ion Channel Coupling?

SigmaR1 binds ion channels from structurally different families such as NMDA and IP3 receptors, Asic, Kv, Nav or Kir channels. However, within these families, SigmaR1 only interacts with specific members. For example, co-immunoprecipitation experiments in MCF7 and liver cells indicate that SigmaR1 binds to IP3R type 1 or 3 but not type 2 [42, 83]; Similarly, Sig1R binds to the GluN1/GluN2A NMDA receptor specifically via the GluN1, but not the GluN2A subunit [51]. These observations raise the question of the structural determinants driving the selectivity of SigmaR1 regarding its partners. Recent studies have point out that transmembrane domains of ion channels could interact with SigmaR1, suggesting that PM lipid microdomain composition may play a central role [43, 84]. Accordingly, we found that SigmaR1:hERG association was reduced in cholesterol-depleted cells. However, the fact that SigmaR1:Asic1 does not depend on PM cholesterol contents suggests that the modalities of interaction may depend on the type of channel chaperoned. Uncovering the molecular mecha-

nisms involved will probably help in understanding the variety of effects produced by SigmaR1 (see Table 6.1). Importantly, the question of the intra-cellular distribution of SigmaR1, which depends on cell type [56, 57] may partly underlie the heterogeneity of regulation mechanisms among pathologies and tissues.

6.4.2 How Do Sigma Ligands Alter SigmaR1:Ion Channel Functional Coupling?

Given that sigma agonists dissociate SigmaR1 from BiP chaperone to promote its binding to IP3 receptor in MAM, it could be speculated that these ligands would also promote SigmaR1:ion channel interaction. This model fits with the results obtained by Kourich et al. in which cocaine and sigma agonists clearly promote K^+ current density by stimulating SigmaR1-dependent trafficking of Kv1.2 (51). However, while SigmaR1 also promotes hERG trafficking, sigma agonists inhibit hERG current density [41], in accordance with a number of studies showing that sigma ligands inhibit SigmaR1-regulated ion channels [4, 15, 40]. These apparent discrepancies between the results obtained in different models (reported in Table 6.1) point out the need for new studies to understand how sigma ligands interact with SigmaR1:ion channel complexes.

6.4.3 SigmaR1 Drives the Dynamic Formation of Channel Signaling Platforms

As described above, SigmaR1 triggers the formation of signaling complexes gathering ion channels and membrane receptors at the PM to enhance cell response to the micro-environment. Consequently, the phenotype related to the presence of SigmaR1 closely corresponds to the phenotype associated to hERG in myeloid leukemia and CRC *in vitro* and *in vivo*. Therefore, the large variety of possible interactions between SigmaR1, ion channel and receptor subtypes among cancers may cover a large diversity of phenotypes shaping disease progression. Considering the fact that SigmaR1 is activated upon stress occurring in injured tissues [9, 58, 85], the chaper one may represent a much more specific target than ion channels which are constitutively active in healthy organs. The validation of this hypothesis will require a wider exploration of the SigmaR1-dependent regulation of channels in different cancer types or within the different stages of disease progression. The use of sigma ligands to repress cancer development will also require a better understanding the effects of sigma agonists in in vivo cancer models. This latter point will benefit from the understanding of the mechanism of action of sigma ligands on SigmaR1:ion channel coupling related above since SigmaR1 agonists promote cell survival in neurodegenerative models but generally inhibit ion channel functions in cancer cells.

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7 Sigma-1 Receptors Fine-Tune the Neuronal Networks

Shang-Yi Anne Tsai and Tsung-Ping Su

Abstract

The endoplasmic reticular (ER) protein sigma-1 receptor (Sig-1R) has been implicated in CNS disorders including but not limited to neurodegenerative diseases, depression, amnesia, and substance abuse. Sig-1Rs are particularly enriched in the specific domain where ER membranes make contacts with the mitochondria (MAM). Within that specific domain, Sig-1Rs play significant roles governing calcium signaling and reactive oxygen species homeostasis to maintain proper neuronal functions. Studies showed that the Sig-1R is pivotal to regulate neuroplasticity and neural survival via multiple aspects of mechanism. Numerous reports have been focusing on Sig-1R's regulatory effects in ER stress, mitochondrial function, oxidative stress and protein chaperoning. In this book chapter, we will discuss the emerging role of Sig-1R in balancing the populations of neuron and glia and their implications in CNS diseases.

Keywords

Glia-neuron interplay • Astrocyte • Axon pathfinding • Axon pruning • Sigma-1 receptor

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7.1 Introduction

Neurons are functionally polarized cells extended with neurites. Among neurites, axons are distinct from other dendrites due to their specialization in conducting signal propagation and protein transport in the neural circuit. Axonal guidance and pathfinding are precisely governed during neuronal developments. Failures or malfunction in axonal maintenance, regeneration and target recognition have been implied in the pathogenesis of several CNS disorders such as Alzheimer's

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disease, Parkinson's disease, stroke and spinal cord injuries [1–3].

The axonal pathfinding in the developing nervous system is orchestrated by cytoskeletal element polymerizations as well as the regulation of microtubule-associated proteins and the Rho-GTPases family. In addition, guidance cues and other stimuli such as extracellular signaling proteins also contribute to the precision of axonal pathfindings. These factors include growth factors, matrix glycoproteins, and integrin receptors. Emerging evidence indicates that local axonal translation plays important roles in axonal maintenance [4, 5]. Many local translational mechanisms for mitochondrial proteins are responsible for preventing free radical production and oxidative damage and thus may be contributing to axonal health [5–7]. Recent reports also indicated that mitochondrial biogenesis is not limited to the cell body, but also occurs locally in axons [8–10].

7.2 The Role of Sig-1R in Neurogenesis and Axon Guidance

We recently discovered that the sigma-1 receptor (Sig-1R), an ER chaperone protein that resides in the ER and mitochondrial contacting site (also known as MAM) [11], is essential for neurogenesis in dentate gyrus of adult hippocampus [12] and is pivotal to maintain dendritic arborization via the regulation of mitochondrial functions during neuronal development [13]. In addition, axon extensions are regulated by Sig-1R activities [14, 15]. In Sig-1R depleted neurons, the growth cones exhibit reduction in size and in Rac GTPase specific GEF Tiam1 intensities. Sig-1R depletion also caused significant reduction in axonal density as well as decreased mitochondrial number and mobility [15]. These findings further support the important notion of Sig-1Rs in maintaining neuronal survival and their implications in many CNS disorders.

In a primary rat hippocampal neuron model, we employed Sig-1R knockdown (KD) using the AAV transduction. Sig-1R deficiency induces non-neuronal cell proliferation as indicated by DAPI staining. Non-neuronal cell proliferation is an early sign of gliosis, and is usually accompanied by astrocytic activation. Axons were visualized by immuostaining with the α-acetylated tubulin. We noticed that the Sig-1R KD neurons exhibited disoriented axon projections (Fig. 7.1). Wild type (WT) hippocampal neurons displayed structurally organized axon networks and connections, while the axons of the KD neurons established abnormal circular routes and displayed a disoriented phenotype. These findings suggest that Sig-1R deficiency may lead to poor arborization of presynaptic axons and fewer synapse formations. Regressive axon growth is essential to coordinate functional axon connections. Axon pruning occurs constantly during axon pathfinding and elongation. Axons may dislocate and mistarget if left without proper pruning. In addition, aberrant axon pathfinding has been associated with neurological diseases [16]. Though Sig-1Rs have been shown to participate in axon elongations [14, 15], surprisingly, Sig-1R antagonists induced aberrant axon elongation in a primary mouse cortical neuron model. 1 μM BD-1063 significantly increased axon elongations in neurons as indicated by phospho neurofilament immunostaining (Fig. 7.2). Similar results were observed using another Sig-1R specific antagonist haloperidol (data not shown). Perhaps it is too early to conclude that inactivation of Sig-1R enhances axonal activities and elongation. Rather, antagonizing Sig-1Rs may disrupt the well-orchestrated mechanisms that are tightly associated with pruning and guidance. This leads to the hypothesis that Sig-1Rs may be involved in axon guidance/pathfinding as well as in axon pruning and facilitate axon targeting to proper functional areas to form functional synapses.

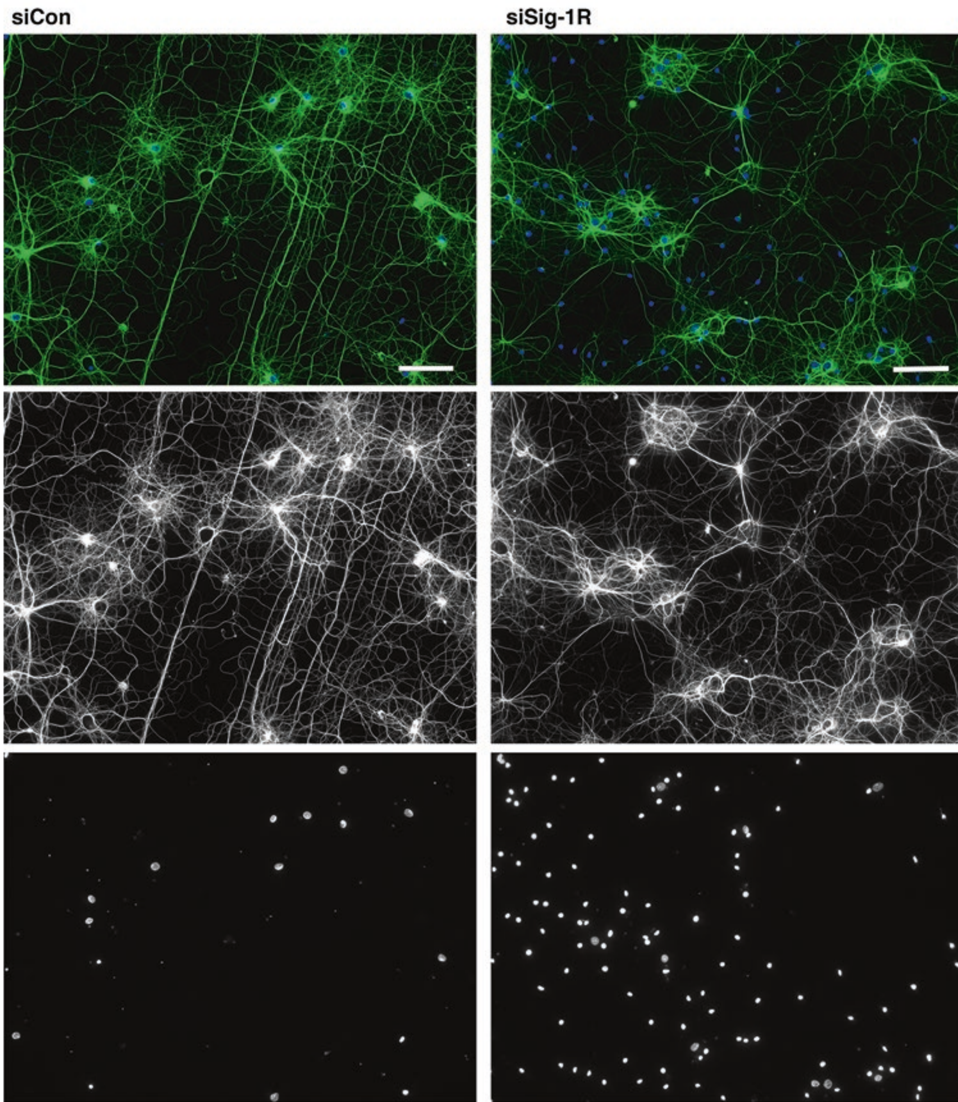

Fig. 7.1 Sig-1R is required to maintain neuronal polarity. Equal density of cultured hippocampal neurons were infected with an AAV vector expressing a short hairpin RNA (shRNA) sequence for Sig-1R. Ten days after transduction, neurons were immunostained with the axon marker acetylated alpha tubulin (*green*). Depletion of Sig-1R disrupts axon polarity and arborization as axons in the Sig-1R KD groups wrapped around neuronal somas and failed to display proper connections. Though the population of neurons in both control and KD groups is similar, Sig-1R KD cultures may be more susceptible to gliosis as indicated by more non-neuronal DAPI staining (*blue*)

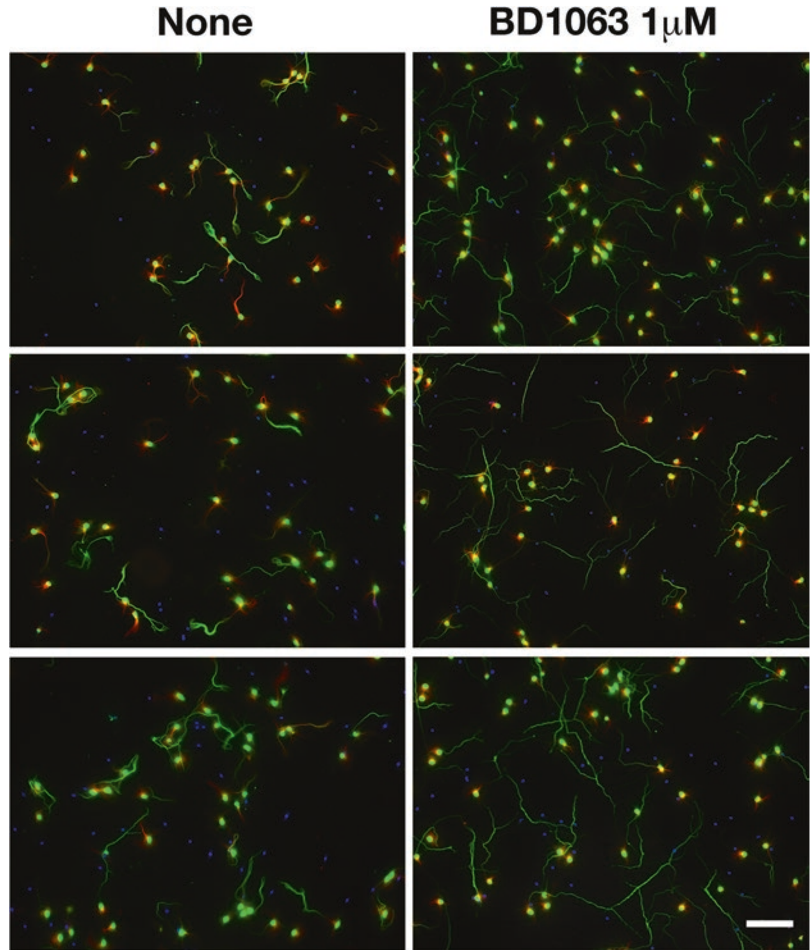

Fig. 7.2 Aberrant axon elongation induced by Sig-1R antagonist. Primary mouse cortical neurons were treated with the Sig-1R antagonist BD-1063 (1 μM) at days in vitro (DIV) 7. Axon lengths were observed at DIV10 by immunostaining of phospho neurofilament (pNF-H, SMI 31). Neurons treated with BD-1063 (*right panels*) showed significantly longer axons than the control neurons (*left panels*)

7.3 Conclusions

Non-neuronal cells are abundant in the central nervous system (CNS) and without doubt participate in axon signaling. Astroglia play important roles and indispensable contributions in many CNS processes including shaping memory formation and recovery from CNS injury. It has been well recognized that the bidirectional astrocyte-neuron communication is part of the axon pruning/pathfinding [17]. A single astrocyte can form synaptic islands by enwrapping a maximum of eight neuron somas and making contact with 300–600 neuronal dendrites [18]. At the synaptic clefts, astrocytes and neurons form the so-called "tripartite synapse" to establish bidirectional communications [19, 20]. Astrocytes can trigger the exocytotic release of gliotransmitters including glutamate, GABA, NMDA receptor co-agonist D-serine and ATP/adenosine, as well as neurotrophic factors [21]. On the other hand, reactive astrocytes can function as the extrinsic inhibition at the lesion site to inhibit axon growth [22, 23]. Sig-1Rs are enriched in astrocytes [24].

Accumulating evidence shows that Sig-1Rs exert regulatory effects on neuropathic pain [25], traumatic brain injury-induced inflammatory responses [26], as well as psychostimulants-induced autophagy [27] and neuroinflammation responses [28] via the astrocytic or microglial activation. Thus, Sig-1Rs may oversee axon guidance/

pathfinding via the precise glia-neuron communication networks (Fig. 7.1) as well as govern the functional axon growth via the mechanisms that regulate recessive events (Fig. 7.2). Sig-1R ligands may exert great therapeutic potentials in establishing functional neuronal networks in this regard.

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Pharmacological Modulation of the Sigma 1 Receptor and the Treatment of Pain

8

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Abstract

There is a critical need for new analgesics acting through new mechanisms of action, which could increase the efficacy with respect to existing therapies and reduce their unwanted effects. Current preclinical evidence supports the modulatory role of sigma-1 receptors (σ_1R) in nociception, mainly based on the pain-attenuated phenotype of σ_1R knockout mice and on the antinociceptive effect exerted by σ_1R antagonists on pains of different etiologies. σ_1R is highly expressed in different pain areas of the CNS and the periphery (particularly dorsal root ganglia), and interacts and modulates the functionality of different receptors and ion channels. The antagonism of σ_1R leads to decreased amplification of pain signaling within the spinal cord (central sensitization), but recent data also support a role at the periphery. σ_1R antagonists have consistently demonstrated efficacy in neuropathic pain, but also in other types of pain including inflammatory, orofacial, visceral, and post-operative pain. Apart from acting alone, when combined with opioids, σ_1R antagonists enhance opioid analgesia but not opioid-induced unwanted effects. Interestingly, unlike opioids, σ_1R antagonists do not modify normal sensory mechanical and thermal sensitivity thresholds but they exert antihypersensitive effects in sensitizing conditions, enabling the reversal of nociceptive thresholds back to normal values. Accordingly, σ_1R antagonists are not strictly analgesics; they are antiallodynic and antihyperalgesic drugs acting when the system is sensitized following prolonged noxious stimulation or persistent abnormal afferent input (e.g., secondary to nerve injury). These are

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distinctive features allowing σ_1R antagonists to exert a modulatory effect specifically in pathophysiological conditions such as chronic pain.

Keywords
Analgesia • Antinociception • Chronic pain • Sigma-1 receptor • Opioid adjuvant • E-52862

8.1 Introduction

Acute pain has evolved as a key physiological alert system for avoiding noxious stimuli and protecting damaged regions of the body by discouraging physical contact and movement [1]. Conversely, chronic pain has been recognized as pain that persists past normal healing time and hence lacks the acute warning function of physiological nociception. Usually pain is regarded as chronic when it lasts or recurs for more than 3–6 months. Chronic pain may be associated with many common diseases or be a disease by itself. It can be debilitating, with those affected typically suffering psychological disturbance and significant activity restrictions. Chronic pain is a frequent condition, affecting an estimated 20 % of people worldwide and accounting for 15–20 % of physician visits [2]. Unfortunately, currently available treatments provide modest improvements in pain and minimum improvements in physical and emotional functioning [3]. Thus, the unmet medical need in the pain research area is huge, and particularly relevant in difficult-to-treat pain modalities, such as neuropathic pain.

Therefore, there is an urgent need to better understand the cellular and molecular mechanisms that mediate chronic pain and to use this knowledge to discover and develop improved therapeutics, especially new drugs acting through new mechanisms of action. Despite very intensive research efforts have translated into exponential growth of pain-related publication productivity and improvements in the understanding of pain mechanisms, those efforts have not yet yielded new analgesics. The most notable therapeutic advances have not been the development of novel evidence-based approaches, but rather changing trends in applications and practices within the available clinical armamentarium. In the absence of real breakthroughs in analgesic drug development, the landscape is dominated by incremental improvements of existing therapies [4], including combination treatments, new formulations of existing drugs, me-too drugs and refinements based on validated mechanisms. Opioids (moderate/severe pain), non-steroidal anti-inflammatory drugs (mild/moderate pain), triptans (migraine), and some anticonvulsants and antidepressants (neuropathic pain) account for the major analgesic classes. Most of them are old or even ancient discoveries and exert modest analgesic effect and/or are limited by their adverse effects, particularly when used chronically [5].

The sigma-1 receptor (σ_1R), a unique ligand-regulated chaperone protein with no precedent and no homology to known proteins [6], has become one among the new and most promising pharmacological targets in pain. Several studies have shown that inhibition of σ_1R leads to decreased amplification of pain signaling within the CNS. Indeed, σ_1R is expressed in several areas of the CNS specialized in nociceptive signaling processing, including the dorsal spinal cord, thalamus, periaqueductal gray (PAG), basolateral amygdala and rostroventral medulla (RVM) [7, 8]. σ_1R is also expressed in peripheral tissues including dorsal root ganglia (DRG) neurons [9, 10]. Importantly, its high density in DRG, in which σ_1R expression is roughly an order of magnitude higher than in several CNS areas involved in pain signaling, points to a functional role of peripheral σ_1R in pain modulation [11]. It is expressed by both sensory neurons and satellite cells in rat DRGs and its expression is downregulated in axotomized neurons and in accompanying satellite glial cells [10].

The use of the σ_1R knockout (KO) mice has been critical to identify the σ_1R as a modulator of

activity-induced sensitization of pain pathways. The σ_1R KO mice is insensitive or shows attenuated expression of pain behaviors in chemically-induced (e.g. formalin, capsaicin) and neuropathic pain models [12–19]. These genetic as well as pharmacological findings using several σ_1R ligands (see [20] for a review) provided evidence to consider σ_1R antagonists as an innovative and alternative approach for treating pain, especially neuropathic pain but also other sensitizing pain conditions. In addition, preclinical evidence has pointed out their potential as an adjuvant therapy to enhance opioid analgesia, without increasing the side effects associated with opioid use [11, 21–23]. As an advantage over opioids, σ_1R antagonists do not alter normal basic pain behavior as they do not modify the normal sensory mechanical and thermal perception in the absence of sensitizing stimuli. That is, σ_1R antagonists exert antiallodynic and antihyperalgesic effects in sensitizing conditions, enabling the reversal of diminished nociceptive thresholds back to normal values, but they do not modify normal sensory thresholds in non-sensitizing conditions, i.e., in normal conditions, in the absence of injury or other inductors of pain hypersensitivity [13, 24–26]. The σ_1R, however, modulates opioid-mediated antinociception in acute non-sensitizing models. σ_1R agonists diminish opioid antinociception whereas antagonists enhance it [21, 27, 28]. As an example, the σ_1R antagonist E-52862 was devoid of activity in the radiant heat tail-flick test but it did potentiate by a factor of 2–3.3 the antinociceptive effect of several opioids, including tramadol, morphine, buprenorphine, codeine, oxycodone, and fentanyl in this acute test. Moreover, E-52862 was effective in restoring antinociception of morphine once tolerance had developed [22].

The purpose of this review is to summarize the current knowledge on the potential of a new drug class, σ_1R antagonists, for the treatment of pain of different etiologies (e.g. neuropathic, inflammatory, visceral, orofacial, postoperative), either used alone or in combination with known analgesics such as opioids**.** Evidence was gained experimentally using genetic approaches, i.e. by the use of σ_1R KO mice or antisense probes, pharmacological tools, experimental drugs in discovery and clinical development phases as well as non-selective marketed drugs. Due to the chaperoning activity of the σ_1R, we have also summarized the current understanding of its interaction with different other molecular targets involved in pain transduction, transmission and processing, to provide some mechanistic background to the observed antinociceptive effects of σ_1R antagonists.

8.2 σ_1R Modulation of Pain Targets

The σ_1R, as a ligand-operated chaperone, is able to interact with other proteins including receptors, enzymes or ion channels, some of which are involved in nociception. Pain is a complex pathology, involving several mechanisms engaging many different molecular targets and intracellular pathways either at central or peripheral nervous system (PNS) [29]. Provided that the σ_1R may act as a chaperone for several of those targets, it can regulate pain at different levels. Here we summarize current knowledge on σ_1R molecular partners in nociception.

8.2.1 Ion Channels

8.2.1.1 Voltage-Gated Sodium Channels

Nociceptors detect noxious stimuli and transmit this sensation to the CNS by means of action potentials, whose generation involves fast inward sodium currents [30]. A direct interaction of σ_1R with neuronal sodium channels has not been described yet, but a physical interaction with the cardiac $Na_v1.5$ has been reported [31]. Both the non-selective σ_1R antagonist haloperidol and the σ_1R agonist (+)-pentazocine have been described to disrupt the $Na_v1.5/\sigma_1$R interaction, haloperidol being more efficacious in reducing this interaction [31]. Accordingly, independent on the agonistic or antagonistic nature of ligands, σ_1R agonists [(+)-SKF-10047 and (+)-pentazocine] and non-selective σ_1R/σ_2R ligands including haloperidol (antagonist) and 1,3-di-o-tolyl-guanidine (DTG) (agonist) all reversibly inhibited

$Na_v1.5$ channels to varying degrees in HEK-293, COS-7 cells and neonatal mouse cardiac myocytes [32]. Patch-clamp recordings in HEK293 cells stably expressing the human cardiac $Na_v1.5$ also revealed inhibitory modulation by some σR ligands, such as (+)-SKF-10047 and dimethyltryptamine (DMT), which was reverted by progesterone to varying degrees, consistent with antagonism of σ_1 and/or σ_2 receptors, and in some cases by σ_1R knockdown with small interfering RNA [33]. Similarly, patch-clamp experiments in isolated intracardiac neurons from neonatal rats revealed that the non-selective σ_1R/σ_2R agonist DTG and the σ_1R selective agonist (+)-pentazocine inhibited voltage-gated sodium channels. The selective σ_1R antagonist BD-1063 did not modulate the current but inhibited DTG block of sodium currents by ~50 %, suggesting that the effects involve, at least in part, σ_1Rs [34]. It is also worth to mention that activation of σ_1R modulates persistent sodium currents in rat medial prefrontal cortex [35], which are a part of the sodium current involved in setting the membrane resting potential in a subthreshold range and hence regulate repetitive firing and enhance synaptic transmission [36]. It has been described that human $Na_v1.8$ channel, a tetrodotoxin-resistant voltage-gated sodium channel expressed by DRGs with a strong implication in pain modulation, displays slower inactivation kinetics and a larger persistent current than already described for this channel in other species [37]. It is tempting to speculate that the interaction of σ_1R described for the $Na_v1.5$ could as well apply for other sodium channels involved in pain, such as $Na_v1.8$ channels, and that its regulation of persistent sodium current in neuronal areas involved in pain could explain part of its role in nociception. Nevertheless, studies investigating the relationship between σ_1R and sodium channels have been hampered by the lack of selectivity of several of the pharmacological tools utilized, thus precluding generalized conclusions. As an example, σ_1R agonists such as (+)-SKF-10047, dextromethorphan and DTG have been found to directly inhibit $Na_v1.2$ and $Na_v1.4$ currents, apparently through a σ_1R-independent mechanism [38].

8.2.1.2 Acid-Sensing Ion Channels

Acid-sensing ion channels (ASICs) are cationic (sodium-permeable) channels activated by extracellular protons, which are responsible for acid-evoked currents in neurons. They are involved in nociception but also in learning, memory and in pathological conditions such as ischemic stroke [39]. A direct interaction between σ_1R and ASIC has been described, which can be modulated by σ_1R ligands. The σ_1R antagonist haloperidol was able to reduce the ASIC1a/σ_1R binding about 50 % [40]. Moreover, σ_1R/ASIC physical interaction has also functional consequences. σ_1R agonists decreased acid-induced ASIC1a currents and intracellular calcium elevations in rat cortical neurons [41], an effect ascribed to σ_1R engagement because the inhibitory effect was counteracted using σ_1R antagonists. In contrast, in ischemic pain induced by hindlimb thrombus, the σ_1R antagonist BD-1047 reduced mechanical allodynia at the periphery synergistically with the ASICs blocker amiloride, whereas the σ_1R agonist PRE-084 induced mechanical allodynia when co-administered with an acidic pH solution, thus suggesting that σ_1R activation facilitates ASICs to promote pain [42].

8.2.1.3 Voltage-Gated Potassium Channels

Potassium channels are also very important players in action potentials driving repolarization. When these channels open, potassium ions cross the membrane to limit neuronal excitability and firing rate. Potassium channels have also been involved in pain [43]. Specific $K_v1.2$ antibodies were shown to co-immunoprecitate the σ_1R in the nucleus accumbens medial shell [44]. This interaction was further confirmed in double transfected NG108–15 cells. $K_v1.2$ is a delayed rectifier channel activated by slight membrane depolarization. In the PNS, $K_v1.2$ are found in the soma and juxtaparanodes of medium-large DRG neurons and are largely decreased after axotomy, which may contribute to the hyperexcitable phenotype (mechanical and cold allodynia) observed after such type of injury [43]. Aydar and colleagues also demonstrated a direct inter-

action of σ_1R with the K_v1.4 in transfected xenopus oocytes and in rat posterior pituitary tissue [45]. σ_1R agonists could elicit a decrease in K_v1.4 conductance in double transfected oocytes, but the co-expression of σ_1R with K_v1.4 resulted in a faster rate of channel inactivation, a reduction in net current efflux and no change in the channel voltage-dependence activation. This ligand independent regulation and the physical interaction with K_v1.4 made Kourrich and colleagues suggest σ_1Rs as auxiliary subunits for voltage-activated potassium channels [44]. An important observation is that K_v1.4 channels are the only K_v1 α subtype expressed in small diameter DRG neurons, meaning that this channel subtype is in charge of potassium conductance in Aδ and C nociceptor fibers [46]. The regulation of this subtype of potassium channel by σ_1R in this particular type of nociceptors is consistent with the regulatory role that σ_1R plays in pain modulation.

8.2.1.4 Calcium-Activated Potassium Channels

Apart from voltage-sensitive potassium channels, σ_1R has been described to regulate non voltage-dependent, small conductance (SK) calcium-activated potassium channels [47]. SK channels activation, secondary to calcium increases after action potentials, produces membrane hyperpolarization to reduce firing frequency of repetitive action potentials [48]. Ca^{2+} entry after synaptic activation opens SK channels that act to limit the amplitude of synaptic potentials and reduce Ca^{2+} influx through the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) [49]. It has also been established that Ca^{2+} influx through NMDAR could open Ca^{2+}-activated K^+ channels in several systems. Using the σ_1R agonist (+)-pentazocine and patch-clamp whole-cell recordings in CA1 pyramidal cells of rat hippocampus, potentiation of NMDAR-mediated responses was found to occur via inhibition of SK channels, that would normally reduce the amplitude of synaptic potentials reducing Ca^{2+} influx through NMDARs [47]. Moreover, the enhanced NMDAR activity was translated into an increased synaptic plasticity as evidenced by a long-term potentiation effect [47]. Another study also found that DTG inhibited SK channel in midbrain dopaminergic neurons and transiently transfected HEK-293 cells, but other σ_1R agonists such as carbetapentane, (+)-SKF-10047 and PRE-084 had no or little effect. The effect of DTG was not affected by high concentrations of the σ_1R antagonist BD-1047, which argue against a coupling of σ_1Rs to SK channels and suggests that DTG directly blocks SK channels [50]. In the absence of further studies it is difficult to know whether σ_1R actually regulates NMDAR via SK channels or it is a ligand- or cell type-dependent finding.

8.2.1.5 Voltage-Gated Calcium Channels

Voltage-gated calcium channels (VGCC) are other ion channels involved in neuronal action potential that contribute to pain pathophysiology [51]. Tchedre and colleagues, based on co-immunoprecipitation studies, proposed the interaction between the σ_1R and the L-type VGCC endogenously expressed in the RGC-5 retinal ganglion cell line [52]. At the functional level, they found that the σ_1R agonist (+)-SKF-10047 inhibited potassium chloride-induced calcium influx in the RGC-5 cell line and calcium currents in rat cultured primary RGCs [52]. Also in retinal ganglion cells, co-localization studies demonstrated that σ_1Rs and L-type VGCCs co-localized and calcium imaging studies showed that σ_1R agonists (+)-SKF10047 and (+)-pentazocine inhibited calcium ion influx through activated VGCCs (L-type). Antagonist treatment using BD-1047 potentiated Ca^{2+} influx through activated VGCCs and abolished inhibitory effects of the σ_1R agonists [53]. Data obtained using rat intracardiac and superior cervical ganglia neurons also revealed that σ_1R ligands decreased calcium channel currents with maximum inhibition ≥95 %, suggesting that σ_1Rs act on all calcium channel subtypes found on the cell body of these sympathetic and parasympathetic neurons, which includes N-, L-, P/Q-, and R-type calcium channels [54]. In addition to affecting a broad population of calcium channel types, σ_1R ligands altered the biophysical properties of these channels (channel inactivation rate was accelerated, and the voltage dependence of both steady-state

inactivation and activation shifted toward more negative potentials). Interestingly, both σ_1R agonists and antagonists depressed calcium channel currents, with a rank order of potency (haloperidol > ibogaine > (+)-pentazocine > DTG) consistent with the effects being mediated by σ_2R and not by σ_1R [54]. Most interestingly, a similar behavior has been described in dissociated rat DRG neurons, as σ_1R agonists (+)-pentazocine and DTG inhibited calcium currents in patch-clamp experiments [55]. The effect was ascribed to σ_1R activation as it was blocked by the σ_1R antagonists BD-1063 or BD-1047. Both (+)-pentazocine and DTG showed similar inhibitory effect on axotomized DRG neurons as they shifted the voltage-dependent activation and steady-state inactivation of VGCC to the left and accelerated VGCC inactivation rate in both control and axotomized DRG neurons. On the contrary, while the antagonist BD-1063 had no effect by itself in normal non-injured DRGs, its application increased calcium currents in the axotomized ones. Pan and colleagues already noticed these paradoxical results, as σ_1R antagonists exert antinociceptive effects while σ_1R agonists are pronociceptive, and it is also known that painful nerve injury is accompanied by reduction of calcium current in axotomized sensory neurons, which in turn results in elevated sensory neuron excitability [55]. Similarly, it should be noted that calcium current inhibition by compounds such as gabapentin or pregabalin is also an antinociceptive strategy. The complexity and heterogeneity of calcium channel signaling throughout neuronal regions involved in pain was argued in order to explain this apparent contradiction. While at the dorsal horn terminals calcium channel activity controls neurotransmitter release and its blockade results in less neurotransmission and hence pain relief, calcium channel inhibition elsewhere (and particularly at the periphery) can result in inhibition of calcium-activated potassium channels that are in control of after-hyperpolarization, membrane excitability, and firing frequency, leading to an opposite final output. That is, lowered inward calcium current has the dominant, overriding effect of decreasing outward current through calcium-activated potassium channels, thus reducing after-hyperpolarization and thereby increasing excitability. Antagonism of sensory neuron σ_1Rs at peripheral sites (including DRGs) may thus relieve pain by rescuing calcium currents required for natural suppression of repetitive firing via opening of calcium-activated potassium channels.

8.2.1.6 Ligand-Gated Calcium Channels

Ligand-gated calcium channels such as glutamate NMDARs also interact with σ_1R. Increased calcium influx through NMDAR and increased level of phosphorylation of these glutamate receptors have been reported following the activation of σ_1R [25, 56, 57]. This increase in the NMDAR phosphorylation state and activity is accompanied by enhanced pain behaviors. Very recently, a direct physical interaction of the σ_1R with the C-terminal of the NMDAR NR1 subunit has been described [58–60] both *in vitro* an *in vivo* using different research approaches. This physical interaction also modulates the cross-talk between opioid analgesia and NMDAR activity [61, 62]. σ_1R activation is pronociceptive, increasing NMDAR activity as explained above. Garzon's group has shown how σ_1R antagonists are able to uncouple the σ_1R-NMDAR association while increasing opioid analgesia and reducing the development of opioid tolerance (Fig. 8.1). All these evidence suggest a role of the σ_1R in the regulation of synaptic plasticity, as NMDAR has been described to mediate different forms of plasticity including long-term potentiation and central sensitization, phenomena linked to forms of pain facilitation such as hyperalgesia and allodynia [63, 64].

8.2.2 G Protein-Coupled Receptors (GPCRs) and Intracellular Second Messenger Machinery

Several G protein-coupled receptors, including targets clearly involved in pain such as the cannabinoid CB_1 and μ-opioid (MOR) receptors [65, 66] have been described as σ_1R partners. Opioids are still the most used analgesics in severe pain

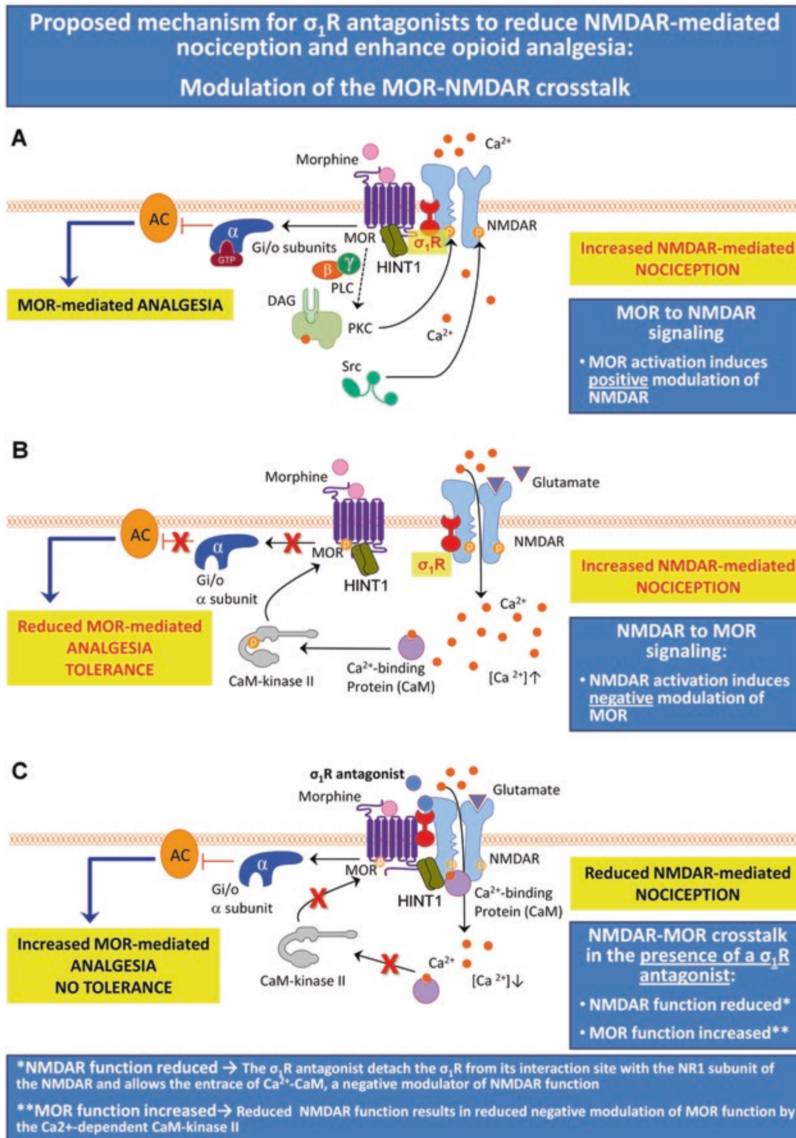

Fig. 8.1 Proposed mechanism for σ_1R antagonists to enhance opioid analgesia based on recent studies reporting modulation of the MOR-NMDAR crosstalk by σ_1R (Rodríguez-Muñoz et al., Antioxidants & Redox Signaling, 2015). σ_1R associates in a calcium-dependent manner with NMDAR NR1 subunits and modulates NMDAR-mediated signaling. Because the σ_1R also associates with the MOR, this protein regulates opioid function within a protein assembly that, via the HINT1 protein, supports MOR-NMDAR physical association and functional cross-regulation. (Panel **a**): MOR to NMDAR signaling: MOR activation induces positive modulation of NMDAR. Upon MOR activation, NMDARs are phosphorylated, increasing their activity and thus NMDAR-mediated nociception. (Panel **b**): NMDAR to MOR signaling: NMDAR activation induces negative modulation of MOR. As a consequence of increased calcium influx through NMDARs, the calcium-calmodulin dependent kinase II becomes activated and phosphorylates MORs, which reduces MOR-mediated analgesia and the response to subsequent morphine challenges (promotes tolerance). (Panel **c**): NMDAR-MOR crosstalk in the presence of a σ_1R antagonist. The absence of σ_1R (e.g. in KO animals) or treatment with a σ_1R antagonist to detach σ_1R from the NMDA NR1 subunit allows the entrance of negative regulators of NMDARs, likely calcium-calmodulin, thus reducing NMDAR function and impairing its negative feedback on MORs. Accordingly, it is proposed that a mechanism by which σ_1R antagonists enhance opioid analgesia is by releasing MORs from the negative influence of NMDARs

conditions [67]. σ_1R modulation of opioid receptors was initially described by Chien and Pasternak [21, 27] demonstrating that σ_1R antagonists potentiate opioid analgesia. At the *in vitro* level, Kim and colleagues demonstrated both a physical, by co-immunoprecipitation experiments, and a functional interaction between MOR and σ_1R in transfected HEK cells. The functional consequences of such an interaction were assessed by means of a GTPγS assay, antagonists increasing opioid efficacy by shifting the EC_{50} values of opioid-induced GTPγS binding by 3- to 10-fold to the left [66]. Cannabinoid receptors also play a role in analgesia and they have been shown to be distributed both in peripheral and CNS regions important for pain transmission [68]. Similarly to MOR, a physical interaction with σ_1R has been described for CB_1 receptors [65]. A functional *in vivo* relationship between these two receptors was demonstrated using the tail-flick test. The NMDAR increased its activity in σ_1R KO mice and it was no longer regulated by cannabinoids as in wild-type (WT) counterparts. Moreover, NMDAR antagonism in the σ_1R KO animals produced no effect on cannabinoid analgesia. Pharmacological intervention showed similar results, because antagonizing σ_1R prevented NMDAR antagonists from reducing CB1 receptor-induced analgesia. For both σ_1R-MOR-NMDAR and σ_1R-CB_1-NMDAR protein complexes, histidine triad nucleotide binding protein 1 (HINT1) has been shown to be another interacting partner. Inhibitors of HINT1 enzymatic activity have been described to enhance morphine-induced analgesia while reducing the development of opioid tolerance [69]. A direct physical interaction between this protein and the σ_1R has been shown recently [65] and the coordinated interaction of HINT1 and σ_1R with NMDAR and its GPCRs partners is able to control the analgesia mediated through those GPCRs (Fig. 8.1). Nociceptors are activated by diverse mediators, such as glutamate, bradykinin, and substance P, which act through GPCRs coupled to Gαq proteins. These Gαq proteins lead to the activation of the phospholipase C (PLC) cascade of intracellular second messengers leading to the release of Ca^{2+} from intracellular stores [70]. The ability of σ_1R to modulate this pathway, and so indirectly GPCRs coupled to the PLC-inositol triphosphate (IP_3)-calcium signaling cascade, represents another link to pain modulation. σ_1R activation has been also shown to stimulate PLC to produce diacylglycerol (DAG) and IP_3 [71] which in turn leads to the activation of IP_3 receptors and efflux of calcium to the cytoplasm. There is growing evidence that σ_1R is an important player at the endoplasmic reticulum (ER) regulating calcium homeostasis. In such a role, σ_1R interacts directly with ankyrin B, BiP or IP_3 receptors [72–74] and ultimately regulates intracellular calcium mobilization from the ER either to the cytosol or to mitochondria in the mitochondria-associated ER membrane (MAM) [74]. σ_1R activation leads to a diminished interaction with ankyrin and BiP, an increase in its interaction with IP_3 receptor and finally a stabilization of this later one facilitating calcium efflux. σ_1R agonists also caused the dissociation of ankyrin B and IP_3 receptors and this activity correlated with the ability of these ligands to potentiate intracellular mobilization induced by bradykinin. This increase in calcium could be reversed by a σ_1R antagonist [75]. Similarly, in CHO cells overexpressing a C-terminal EYFP tagged σ_1R, agonists, such as (+)-pentazocine and PRE-084, caused significant uncoupling of the σ_1R-BiP complex, whereas antagonists, such as NE-100 or haloperidol, were not able to modify that complex at all [73].

8.2.3 Homomerization

Finally, σ_1R interacts with itself [76, 77]. A GXXXG motif of the σ_1R is involved in the oligomerization process, as mutations of this σ_1R region reduced the number of receptors in higher oligomeric states and favored smaller oligomeric ones [78]. Moreover, only oligomeric and not the monomeric forms of σ_1R could bind the specific agonist (+)-pentazocine. Another finding by Gromek and colleagues was that ligand binding to σ_1R oligomers could prevent the formation of the monomer form, emphasizing the important role that σ_1R oligomers have on its pharmacology [78].

Thus, pharmacological activity of σ_1R ligands, including their pro- or antinociceptive activities, could be at least in part consequence of their influence in regulating and/or interacting with σ_1R oligomeric states.

8.3 Sigma-1 Receptor Antagonism as a New Analgesic Strategy

8.3.1 Synthetic Sigma-1 Receptor Antagonists

Many structurally diverse compounds bind to the σ_1R (agonists, Fig. 8.2 and antagonists, Fig. 8.3). Several compounds have undergone clinical trials, but only E-52862 is being developed for pain indications. In fact, no selective σ_1R ligands have so far been marketed, although many drugs on the market show affinity for the σ_1R [20].

While a long list of xenobiotic compounds interact with the σ_1R, there are few known endogenous small molecules showing binding affinity to the receptor. Endogenous compounds that have been proposed as putative endogenous σ_1R ligands include neurosteroids, some sphingolipids and dimethyltryptamine (Figs. 8.2 and 8.3). Their exact physiological roles in the context of the modulation of σRs are still not clear, but it is remarkable that none of them show high affinity for the σ_1R and only one, progesterone, is described as a σ_1R antagonist.

Clinically used drugs with an affinity for the σ_1R include drugs with different therapeutic applications, such as antipsychotics (haloperidol: D2/D3 antagonist), antidepressants (fluvoxamine, sertraline, fluoxetine, imipramine: SSRI

σ_1R agonists

Pharmacological tools

- **PRE-084** (Ki nM: σ_1=2.2; σ_2=13091)
- **(+)-Pentazocine** (Ki nM: σ_1=16.7; σ_2=6611)
- **DTG** (Ki nM: σ_1=203; σ_2=58.4)
- **(+)-SKF-10,047** (Ki nM: σ_1=597; σ_2=39740)

Su et al., 1991
Vilner and Bowen, 2000

Putative endogenous ligands

- **L-threo-sphingosine** (Ki nM: σ_1=20; σ_2=8300)
- **Sphinganine** (Ki nM: σ_1=70; σ_2=3500)
- **N,N-dimethylsphingosine** (Ki nM: σ_1=120; σ_2=2800)
- **D-erythro-sphingosine** (Ki nM: σ_1=140; σ_2=13000)
- **PREGS** (Ki nM: σ_1=3200; σ_2:ND)
- **DMT** (Ki nM: σ_1=14750; σ_2=21710)

Ramachandran et al., 2009
Su et al., 1988
Fontanilla et al., 2009

Drugs under active development in clinical trials

- **Cutamesine (SA4503)** (Ki nM: σ_1=4.6; σ_2=63.1): STROKE
- **Anavex 2-73** (IC50 nM: σ_1=860; σ_2: ND): ALZHEIMER

Lever et al., 2006
Lahmy et al., 2013

Drugs in the market with affinity for the σ_1R / Non selective ligands

- **(+/-)-Pentazocine** (Ki nM: σ_1=12.1; σ_2=1880)
- **Carbetapentane** (Ki nM: σ_1=129; σ_2=1953)
- **Dextromethorphan** (Ki nM: σ_1=205; σ_2=11060)
- **Donepezil** (Ki nM: σ_1=14.6; σ_2:ND)
- **Fluvoxamine** (Ki nM: σ_1=36; σ_2=8439)
- **Sertraline** (Ki nM: σ_1=57; σ_2=5297)
- **Fluoxetine** (Ki nM: σ_1=240; σ_2=16100)
- **Imipramine** (Ki nM: σ_1=343; σ_2=2107)

Kozaka et al., 2012
Calderon et al., 1994
Shin et al., 2005
Kato et al., 1999
Narita et al., 1996

Drugs discontinued in clinical trials / No recent development reported

- **Igmesine** (Ki nM: σ_1=75; σ_2>1000) (discont. 2000, Phase III)
- **Siramesine** (IC50 nM: σ_1=17; σ_2=0.12) (discont. 2002, Phase II)
- **SR-31747A** (IC50 nM: σ_1=4.2; σ_2=45) (discont. 2007, Phase II)

Mainly explored for the treatment of major depressive disorder and anxiety disorder

Sorbera et al., 1999
Perregaard et al., 1995
Bourrié et al., 2002

Fig. 8.2 σ_1R agonists

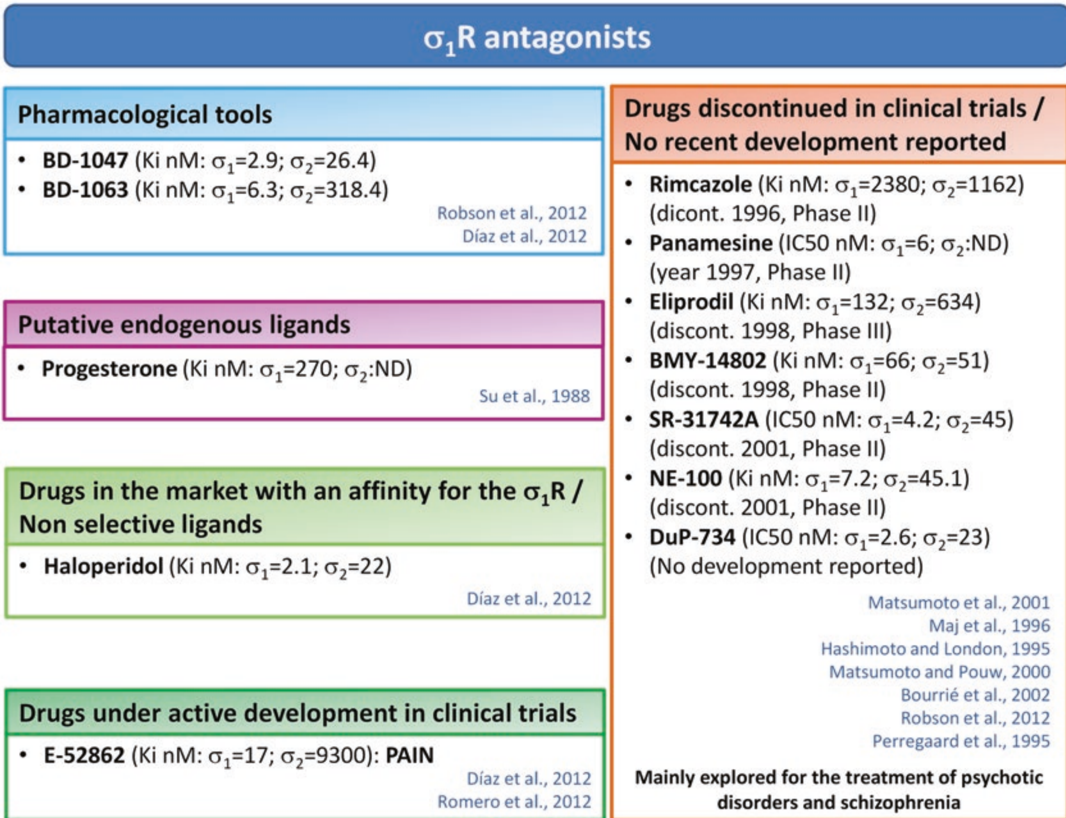

Fig. 8.3 σ_1R antagonists

and non-SSRI), analgesics (pentazocine: opioid agonist), antitussives (carbetapentane: muscarinic antagonist, dextromethorphan: NMDA antagonist) and drugs for the treatment of neurodegenerative disorders such as Alzheimer's disease (donepezil: cholinesterase inhibitor). All of these drugs can bind to σ_1R with high to moderate/weak affinity, but none of them show selectivity over other main therapeutic targets. Haloperidol acts as a σ_1R antagonist, whereas fluvoxamine, sertraline, fluoxetine, imipramine, pentazocine, carbetapentane, dextromethorphan and donepezil act as σ_1R agonists (see [6] for a review). In spite of their lack of selectivity, several of these compounds have been used as pharmacological tools in understanding the role of the σ_1R in pain. Details on their activities in different pain models can be found in [6] and are also briefly described in the following sections.

Since the mammalian and human σ_1Rs were cloned in 1996 [79, 80], new high affinity ligands for the σ_1R have been developed. In the 1990s and in early 2000s some σR ligands reached Phase II clinical trials for the treatment of neuropsychiatric disorders, but most of them did not progress up to Phase III. No information on their clinical use in pain is available. Proposed σ_1R agonists discontinued in clinical development (Fig. 8.2) include: igmesine (Phase III; depression and Alzheimer's disease; Pfizer Inc.), siramesine (Phase II; anxiety disorder; H Lundbeck A/S and Forest Laboratories Inc.), SR-31747A (Phase II; rheumatoid arthritis and cancer; Sanofi-Synthelabo). Proposed σ_1R antagonists discontinued in clinical development (Fig. 8.3) include: rimcazole (Phase II; psychotic disorder; GlaxoSmithKline), panamesine (Phase II; psychotic disorder and schizophrenia; Merck KGaA),

eliprodil (Phase III; head injury and stroke, Synthelabo and Lorex Pharmaceuticals Inc), BMY-14802 or BMS-181100 (Phase II; psychotic disorder and schizophrenia; Bristol-Myers Squibb Co), SR-31742A (Phase II; psychotic disorder and schizophrenia; Sanofi-Synthelabo), NE-100 (Phase II; psychotic disorder and schizophrenia; Taisho Pharmaceutical Co Ltd) and DuP-734 (No development reported; psychotic disorder and schizophrenia; Bristol-Myers Squibb Pharma Co). As recently reviewed [6, 20], these compounds were defined as σ_1R ligands, but information on both the molecular structure of the σ_1R and structural, functional-determining features of σ_1R ligands was very poor at that time. Many of them were not selective versus σ_2R and/or other molecular targets. In addition, a number of them showed suboptimal metabolic profiles or were highly lipophilic, reasons that may have affected their potential development. Thus, past clinical failures do not preclude a potential role of σ_1R modulation in the above cited indications.

Only recently, more selective and optimized compounds have become available for the accurate assessment of the σ_1R as a therapeutic target. Since 2006, some σ_1R ligands have been extensively studied for their potential in treating both acute and chronic neurodegenerative diseases and neuropathic pain. σR ligands commercially available and used as pharmacological tools include PRE-084, (+)-pentazocine, DTG and (+)-SKF-10,047 as agonists (Fig. 8.2); and BD-1047, BD-1063 and NE-100 as antagonists (Fig. 8.3). Although they have been very useful to ascertain the role of the σ_1R in pain, some of them are still not selective enough to draw definitive conclusions, and sometimes paradoxical or inconsistent results have been reported. Details on their activities in different pain models can be found in [6, 20], and are also briefly described next in this chapter.

To date, three pharmaceutical companies, Anavex Life Sciences Corp. (with the σ_1R agonist Anavex 2–73), M's Science Corp. (with the σ_1R agonist cutamesine) and ESTEVE (with the σ_1R antagonist E-52862 or S1RA) are actively engaged in clinical trials of σ_1R ligands. The R&D team of ESTEVE disclosed a wide series of compounds with affinity for the σ_1R, selecting E-52862 for clinical development. E-52862 has been a very useful tool to assess the role of the σ_1R in pain, as it shows high affinity for the σ_1R (Ki = 17 nM) and has high selectivity over the σ_2R and many other molecular targets [26]. In the recent years, E-52862 (many times identified as S1RA) has been used to explore the potential of σ_1R antagonists in pain indications of different etiology, as well as in understanding the mode of action of this new class of drugs [11, 15, 18, 19, 21–23, 26, 81–83]. The safety and pharmacokinetic profile of E-52862 were studied in a rigorous Phase I program, showing favorable safety results at all doses tested [84, 85]. Today, the E-52862 clinical program focuses on pain management as opioid-adjuvant therapy and as monotherapy in several neuropathic pain conditions, including diabetic-, post-operative-, and chemotherapy-induced neuropathic pain.

8.3.2 Sigma-1 Receptor Modulation of Opioid Analgesia

Opioids are the gold standard painkillers used for the treatment of moderate to severe pain. Although they are used worldwide, they exert well-known side effects that limit their use such as constipation, dizziness and nausea, among others, which usually lead to treatment discontinuation [86]. Other side effects, such as tolerance and dependence appear in long-term treatments. Consequently a reduction in treatment effectiveness and increase consumption are normally associated with opioids use, increasing the risk of death from multiple causes compared with non-users [87]. Thus, in order to minimize opioid-related adverse events, several approaches combining other drugs with opioids to increase their potency and consequently reduce the opioid doses, have been proposed.

A relationship between the σR system and opioid analgesia was described more than 20 years ago by Chien and Pasternak. They showed that σ_1R agonists counteracted opioid receptor-mediated analgesia, while σ_1R antagonists

potentiated it [21, 24, 27, 88]. The systemic administration of (+)-pentazocine or DTG (σ_1R agonists) inhibited whereas haloperidol (D2 receptor and σ_1R antagonist) enhanced morphine antinociception in the tail-flick test in mice and rats [21, 24]. The enhancing effect of haloperidol was mediated by σ_1R blocking, since (−)-sulpiride (selective D2 receptor antagonist) was unable to potentiate opioid analgesia [21, 27]. The actions of σ_1R ligands were not limited to the modulation of morphine analgesia. Treatment with σ_1R receptor ligands modulates the antinociception induced by other μ-, δ and κ-opioid receptor agonists, such as D-penicillamine-2-D-penicillamine-5-enkephaline, U-50488H, nalbuphine or naloxone benzoylhydrazone [21, 24, 28, 89, 90]. The modulation of opioid analgesia by σ_1R ligands was later supported by studies using other σ_1R agonists ([+/−]-PPCC) and antagonists ([+]-MR200, compound 9, BD-1063 or E-52862) [22, 90–93] as well as σ_1R antisense oligodeoxynucleotides [28, 89, 94, 95].

Altogether, data support the presence of an endogenous σ_1R system, tonically active, whereby σ_1R exerts a tonic inhibitory control on the opioid receptor-mediated signaling pathways. This endogenous system can be pharmacologically counteracted by using σ_1R antagonists to increase the response to opioids. This pharmacological interaction has been supported by molecular studies, already described in this review (see σ_1R modulation of pain targets section and Fig. 8.1). σ_1R antagonists enhance opioid analgesia in naïve mice by releasing MORs from the negative influence of NMDARs, and even more, they also reset antinociception in morphine-tolerant animals [60], which support a previous result with systemically administered drugs where the σ_1R antagonist E-52862 restored morphine-induced antinociception in morphine tolerant mice [22].

Regarding the site of action, the modulation of opioid-induced antinociception has been observed both at peripheral and central (mainly supraspinal) levels, suggesting that σ_1R-mediated pain modulation occurs at different sites [11, 22, 23]. The supraspinal site of action of σ_1R was firstly demonstrated by the use of the σ_1R agonist (+)-pentazocine microinjected in periaqueductal gray, locus coeruleus, or RVM. It diminished systemic opioid analgesia in the tail-flick model in mice. In turn, the σ_1R antagonist haloperidol and also antisense oligonucleotides microinjected into the RVM markedly enhanced the analgesic actions of co-administered morphine. On the contrary, σ_1R agonists spinally administered did not alter opioid analgesia [28, 95].

A peripheral site of action of σ_1R in the modulation of opioid-induced antinociception has been recently reported by using the paw pressure test in mice [11, 23]. BD-1063, BD-1047, NE-100 and E-52862 were devoid of effect in mechanical nociception when administered locally (intraplantarly). However, these σ_1R antagonists markedly potentiated opioid antinociception of an inactive dose of morphine, their effects being reversed by the selective σ_1R agonist PRE-084 [23]. In addition, σ_1R KO mice exhibited an enhanced mechanical antinociception in response to morphine (local or systemic) [23]. Similar findings were observed using other opioids such as fentanyl, oxycodone, buprenorphine, tramadol or even the peripheral opioid loperamide [11]. The peripheral component of the enhancement of opioid antinociception by σ_1R antagonists was also evidenced by using the radiant heat tail-flick test in rats [96]. In this study, the systemic administration of peripheral opioid agonist loperamide was devoid of antinociceptive effect when given alone but produced antinociception when combined with E-52862. Accordingly, the antinociceptive effect of the combination was abolished by the systemic administration of the peripheral opioid antagonist naloxone methiodide.

It is worthwhile that the increase in opioid potency by σ_1R antagonists co-administration appears to be limited to analgesia and not to side effects. E-52862 enhanced by a factor of 2–3.3 the antinociceptive effect of several opioids in the tail-flick test, including tramadol, morphine, buprenorphine, codeine, oxycodone, and fentanyl. The antinociceptive effect was attributed to the σ_1R, provided that E-52862 was devoid of potentiation effect on morphine analgesia in mice lacking σ_1R. However, morphine-induced antinociceptive tolerance and rewarding were attenuated whereas physical dependence, inhibition of

gastrointestinal transit, or mydriasis were not modified [22]. Finally, in addition to opioid analgesia, the σ_1R antagonist BD-1047 has been shown to potentiate clonidine analgesia without affecting the motor impairment produced by the alpha-2 adrenoceptor agonist in the mouse orofacial formalin model [97], thus suggesting the possibility that the σ_1R system could be modulating other antinociceptive systems different from opioids.

In summary, σ_1R antagonists have been shown to systemically and peripherally potentiate opioid analgesia but not opioid-related adverse effects, which suggest an application for σ_1R antagonists as opioid adjuvant therapy.

8.3.3 Sigma-1 Receptor Antagonists for the Treatment of Neuropathic Pain

Neuropathic pain has been defined by the IASP (International Association for the Study of Pain) as "Pain caused by a lesion or disease of the somatosensory nervous system, either peripheral or central". This type of pain is chronic and can be extremely severe and crippling for the individual. Neuropathic pain is described by patients as a persistent, diffuse, burning-like sensation with no specific location in a given organ or tissue. In addition, they suffer from paroxysmal pain, that is, short electric shock-like sensations alternating with remission periods. Neuropathic pain is one of the most challenging types of pain because effective and safe neuropathic pain treatment remains a largely unmet therapeutic need [98]. Neuropathic pain patients show general insensitivity to non-steroidal anti-inflammatory drugs (NSAIDs) and relative resistance to opioids. Moreover, some of these drugs involve dose limitations with respect to efficacy and side effects.

Studies using σ_1R KO mice and new selective σ_1R antagonists have identified the σ_1R as a key participant in the modulation of pain behavior in sensitizing and chronic pain conditions, supporting the use of the selective σ_1R antagonists for the treatment of neuropathic pain [93]. σ_1R KO mice are a useful genetic tool to study the involvement of σ_1R in several pain types, provided that KO mice perceive and respond normally to stimuli of different nature (mechanical, chemical and thermal). Thus, the absence of σ_1R in KO mice has been shown to not interfere with the perception of several stimuli applied to the hind paw or with the motor response required for paw withdrawal [12, 14–16, 26]. In σ_1R KO mice, both phases of formalin-induced pain were clearly reduced [12] and capsaicin injected intraplantarly did not induce mechanical allodynia [13]. Regarding neuropathic pain models, cold and mechanical hypersensitivity were strongly attenuated in σ_1R KO mice treated with paclitaxel (concomitant with paclitaxel-induced sensory nerve mitochondrial abnormalities) [15] or exposed to partial sciatic nerve ligation (PSNL) [14], supporting a role of this receptor in the development of the neuropathic pain.

σ_1R antagonists administered alone fail to modify pain by themselves in classical models of thermal and mechanical acute nociception, as seen in the tail-flick, the hot plate and the paw pressure tests in rodents [14, 23, 92]. However, when σ_1R antagonists are administered in sensitizing and chronic pain models they produce similar results as those described in the σ_1R KO mice. The σ_1R antagonist haloperidol, its metabolites I and II and E-52862 inhibited formalin-induced pain [26, 99] and capsaicin-induced sensitization in mice [26, 100]. Pain-related behaviors have also been reversed using σ_1R antagonists in neuropathic pain models in mice, such as the chronic compression of the DRG [101], PSNL [26] and paclitaxel-induced neuropathic pain [15], among others. In an operant self-administration model, mice with PSNL, but not sham-operated animals, self-administered E-52862. In addition, an anhedonic state (decrease in the preference for 2 % sucrose solution) was revealed in nerve-injured mice, which was attenuated by E-52862. Thus, it was concluded that E-52862 showed antinociceptive efficacy following nerve injury associated with an improvement of the emotional negative state

and was devoid of reinforcing effects [82]. Paradoxically, some studies have reported antinociceptive activities in neuropathic pain related to σ_1R agonist activity [102, 103]. The σ_1R agonist (+)-pentazocine acutely injected into the dorsal surface of the hindpaw produced an antinociceptive effect on mechanical allodynia induced in streptozotocin-induced diabetic mice. The effect was inhibited by local hindpaw pretreatment with the σ_1R receptor antagonist BD-1047 in the same area [102]. The authors suggested that the antinociceptive effect of (+)-pentazocine involves lowering of nitric oxide (NO) metabolites in the hindpaw and was discussed as a possible dose effect (peripheral application of the σ_1R agonist (+)-pentazocine could produce the nociceptive response at lower dose, whereas, at higher doses as used in the study, it produces the antiallodynic effect). Attenuation of calcium channel currents involved in peripheral nerve transmission was also discussed as a possible underlying mechanism for the antiallodynic, local, peripheral effect of (+)-pentazocine. In this sense, the σ_1R agonist SA-4503, but not the σ_1R antagonist NE-100, was found to produce antinociceptive effects against chemotherapeutic-induced neuropathic pain in rats [103]. The reasons for these apparent discrepancies are not clear, but the categorization of σ_1R ligands as agonists or antagonists is still unclear and several factors, including drug concentration, site of application, readouts, and diverse experimental conditions could account for these differences.

Several studies have reported changes in σ_1R expression in some phases of the experimental neuropathic models, further supporting the involvement of the σ_1R in the development of the neuropathic pain. σ_1R expression is up-regulated in the spinal cord during the induction phase of neuropathic pain following sciatic nerve constriction or chronic compression of the DRG [57, 101, 104] and in the brain 10 weeks after the induction of diabetic neuropathy [105]. However, the expression of σ_1R was reduced in the spinal cord following chemotherapy (oxaliplatin and paclitaxel) treatment [103] and in DRGs following spinal nerve ligation [10]. Thus, a general rule on how σ_1R expression is modified in neuropathic pain conditions cannot be established.

σ_1R has been involved in the activation of the extracellular signal-regulated kinase (ERK) in the spinal cord in neuropathic pain models such as chronic constriction compression of the DRG, PSNL, and paclitaxel-induced neuropathic pain [14, 15, 101]. In particular, ERK phosphorylation within the spinal cord has been associated with mechanical and cold allodynia in animal models of neuropathic pain. Accordingly, σ_1R KO mice, that exhibited reduced cold allodynia and did not develop mechanical allodynia as compared to WT mice, showed reduced ERK phosphorylation in the spinal cord [14, 15].

ERK activation feeds back on the NMDAR by increasing the expression and phosphorylation status of its NR1 subunit, leading to NMDAR over-activation during neuropathy. It is known that the σ_1R plays an important role in modulating NMDA activity because: (i) pain-related NR1 phosphorylation and expression increase are enhanced by σ_1R agonists and blocked by σ_1R antagonists [25], (ii) σ_1R is physically associated with NMDAR and control its negative influence on MOR [60], and (iii) σ_1R ligands showing no affinity for NMDAR were found to modulate NMDA-induced Ca^{2+} influx and NMDA-induced neuronal activity [56]. Therefore, a picture emerges whereby σ_1R modulates the activity of spinal NMDA receptors, which in turn regulate plastic adaptations associated with central sensitization. In this context, σ_1R antagonists counteract NMDAR activation.

In agreement with these results, the spinal wind-up response after repeated stimulation of C fibers is reduced in σ_1R KO mice and after the administration of σ_1R antagonists to WT mice, which is indicative of the role played by σ_1R in mechanisms underlying central sensitization and synaptic plasticity [14, 26, 83].

Altogether, these findings highlight σ_1R as a new constituent of the mechanisms modulating activity-induced sensitization in nociceptive pathways and thus as a new potential target of action for drugs designed to alleviate neuropathic pain.

8.3.4 Sigma-1 Receptor Antagonists for the Treatment of Inflammatory Pain

Inflammatory pain is largely treated with non-steroidal anti-inflammatory drugs (NSAIDs), acetaminophen, opioids and steroids. These agents may also be used in combination depending on the nature and chronicity of the disease. The acute inflammatory response is controlled relatively efficaciously with these drugs, however in the inflammatory pain associated with chronic diseases, such as rheumatoid arthritis, osteoarthritis or cancer, these drugs are of limited usefulness and thus a significant unmet clinical need for the treatment of chronic inflammatory pain remains.

Recently, a possible role for σ_1R in inflammatory pain has been suggested in different animal models using σ_1R KO mice and ligands (see [106] for review). The genetic inactivation of σ_1R did not alter the development of carrageenan (CARR)-induced and Complete Freund Adjuvant (CFA)-induced behavioral hypersensitivity [18]. However, pain-like responses evoked by a blunt mechanical stimulus were inhibited in the CARR-sensitized σ_1R KO mice [19]. These data indicated that the role of σ_1R on the development of behavioral hypersensitivity induced by peripheral inflammation could vary depending on the experimental conditions, especially the behavioral endpoint analyzed. Furthermore, since behavioral hypersensitivity, especially after mechanical stimulation, is attenuated in animal models of neuropathic but not inflammatory pain, a differential role for σ_1R depending on the etiology of pain (neuropathic *versus* inflammatory) is also suggested. This is not surprising since neuropathic and inflammatory pains are known to involve different pathways. Whereas the decrease in the pain threshold in inflammatory pain is due to the production of pro-inflammatory mediators, such as bradykinin, prostaglandins, leukotrienes, serotonin, histamine, substance P, thromboxanes, adenosine and ATP, protons, free radicals and cytokines [107], neuropathic pain is primarily due to direct damage of peripheral nerves, causing the continuous activity of the nociceptive fibers and subsequent peripheral and central sensitization phenomena. As mentioned in the previous section, ERK phosphorylation is a key process involved in pain sensitization pathways, the increased pERK levels in the dorsal spinal cord during neuropathy being attenuated in σ_1R KO, or after σ_1R pharmacological inhibition. However, the pain-related hypersensitivity observed in WT mice 3 h after CARR [19] or 4 days after CFA injection (data not published obtained in our laboratory), was not accompanied by a selective increase in ERK phosphorylation within the spinal cord. These results not only support the involvement of different mechanisms in the sensory hypersensitivity of experimental models of inflammatory and neuropathic pain, but also that mechanisms by which the σ_1R regulates nociception may be also different.

Regarding σ_1R ligands, the systemic and peripheral administration of different σ_1R antagonists blocked the behavioral hypersensitivity in animal models of inflammatory pain. The antihypersensitivity effect provided by E-52862 was similar to that of ibuprofen and celecoxib in both acute (CARR) and chronic (CFA) pain models. The effect was attributed to the σ_1R provided that E-52862 was devoid of effect in σ_1R KO mice [18]. Unlike anti-inflammatory agents, σ_1R antagonists exert antinociceptive but not anti-inflammatory activity, as the CARR-induced edema remained unaffected in σ_1R KO mice or after treatment with E-52862 or BD-1063 in WT mice [18, 19]. Other σ_1R antagonists, such as (−)-MRV3 and (+)-MR200 have been tested in the CARR model in rats. A dose-dependent inhibition of mechanical allodynia and thermal hyperalgesia was again observed. However, in this case, a significant reduction of the CARR-induced edema was reported with these ligands [108, 109]. Finally, a recent study describes that *N*-(2-morpholin-4-yl-ethyl)-2-(1-naphthyloxy) acetamide (NMIN) and BD-1063 were effective in the chronic constriction injury neuropathic pain model but not in the arthritic pain-induced functional impairment model in the rat [110], further suggesting a differential role of the σ_1R

depending on the type of pain, experimental conditions, and readouts.

The molecular mechanisms underlying the antinociceptive effect of σ_1R antagonists in inflammatory pain have been only partially explored. The inhibition of inflammation-induced spinal sensitization in both neurons, measured as immunoreactivity to Fos, PKC, and PKC-dependent phosphorylation of NR1, and microglia, measured as inhibition of p-p38 mitogen-activated protein kinase (MAPK) and IL-1β immunoreactivity, has been recently suggested to explain the antinociceptive effect of BD-1047 in the zymosan-induced thermal and mechanical hyperalgesia [111]. Other possible mechanisms include the modulation of bradykinin-induced Ca^{2+} release [75] and NO signaling [112], both key mediators released during inflammation and contributing to the peripheral sensitization, which are enhanced by σ_1R activation.

Regulating excitability of peripheral afferents is being pursued as a possible strategy to manage pathological pain [113, 114]. This "peripheral strategy" is of particular interest because of the potential of developing novel drugs that do not access central sites, or to deliver drugs locally by topical or other application methods. Both approaches avoid central exposure to drugs and have thus the potential to reduce side effects compared to systemic administration of drug crossing the blood-brain barrier. The role of peripheral σ_1R in inflammatory pain has been recently studied by Tejada et al. [19]. These authors have identified peripheral σ_1Rs as a key sites contributing to the antinociceptive effect of σ_1R antagonists to ameliorate inflammatory hyperalgesia. They found that intraplantar administration of several σ_1R antagonists in the inflamed paw was sufficient to completely reverse hyperalgesia and that the σ_1R agonist PRE-084 blocked the systemically-induced antinociceptive effect of selective σ_1R antagonists in the CARR pain model. The role of peripheral σ_1R is supported by its high density in DRGs [11]. The contribution of the peripheral σ_1R in types of pain other than inflammatory merits further studies.

8.3.5 Sigma-1 Receptor Antagonists for the Treatment of Other Types of Pain

8.3.5.1 Visceral Pain

Visceral pain is the most frequent type of pathological pain and one of the main reasons for patients to seek medical assistance [115]. However, most of our knowledge about pain mechanisms derives from experimental studies of somatic (principally cutaneous) pain rather than visceral pain. The associated symptoms, pathophysiological mechanisms, and response to drug treatment of visceral and somatic pain are different; consequently, it is not valid to indiscriminately extrapolate findings from the somatic–cutaneous to the visceral domain [116]. In spite of its importance, very few papers have addressed the role of σ_1R in visceral pain. In this regard, González-Cano and co-workers [16] evaluated the role played by σ_1R in the intracolonic capsaicin-induced visceral pain model, measuring both pain-related behaviors and referred mechanical hyperalgesia to the abdominal wall. The intracolonic administration of capsaicin induced concentration-dependent visceral pain-related behaviors and referred hyperalgesia in both WT and σ_1R-KO mice, but the maximum number of pain-related behaviors induced by 1 % capsaicin was roughly 50 % in the σ_1R-KO mice compared to the WT. Several σ_1R antagonists (BD-1063, E-52862 and NE-100) administered subcutaneously dose-dependently reduced the number of behavioral responses and reversed the referred mechanical hyperalgesia to control thresholds in WT mice. These compounds were inactive in the σ_1R-KO mice, thus confirming the σ_1R-mediated effect.

8.3.5.2 Orofacial Pain

Some of the most prevalent and debilitating pain conditions arise from the structures innervated by the trigeminal system (head, face, masticatory musculature, temporomandibular joint and associated structures) [117]. Orofacial pain disorders are highly prevalent and debilitating conditions involving the head, face, and neck. These condi-

tions represent a challenge to the clinician since the orofacial region is complex and pain can arise from many sources. According to Okeson [118], orofacial pain is divided into physical and psychological conditions. Physical conditions comprise: (i) temporomandibular disorders, which include disorders of the temporomandibular joint and disorders of musculoskeletal structures (e.g., masticatory muscles and cervical spine); (ii) neuropathic pains, which include episodic (e.g., trigeminal neuralgia) and continuous (e.g., peripheral/centralized mediated) pains; and (iii) neurovascular disorders, including migraine. Psychological alterations include mood and anxiety disorders.

The role of σ_1R in orofacial pain has been addressed by Kwon et al., who described attenuation of pain behavior (face grooming) after BD-1047 administration in a model of headache pain induced by intracisternal capsaicin administration in rats [119]. Moreover, the σ_1R antagonist BD-1047 consistently reduced capsaicin-induced Fos-like immunoreactivity and the phosphorylation of the NR1 subunit of the NMDAR in the trigeminal nucleus caudalis (TNC) in a dose-dependent manner. As intracranial headaches, including migraines, are mediated by nociceptive activation of the TNC, the authors propose that the use of σ_1R antagonists may be a promising strategy for the treatment of headache disorders. In the same way, Pyun et al. reported that chronic activation of σ_1R by intracisternal administration of the σ_1R agonist PRE084 produced TNC neuronal activation as a migraine trigger in rats. Accordingly, chronic (over 7 days) intracisternal injection of PRE-084 produced sustained neuronal activation (measured as Fos and ΔFosB immunoreactivity) accompanied by increased neuronal susceptibility (measured as phosphorylation of the NMDAR and ERK) in the TNC, which correlated with an increase in face grooming/scratching behavior [120]. The authors pointed out the possible role of neurosteroids in migraine triggering in humans, as migraine is three times more common in women than in men, and frequently evokes pain during the low progesterone peri-menstrual phase [121]. Consistently, systemic injection of the σ_1R antagonist progesterone reduced migraine symptoms in both humans and animals [122, 123], whereas other neurosteroids behaving as σ_1R agonists, including dehydroepiandrosterone, have a pronociceptive role [124].

Roh et al. showed that intraperitoneal BD-1047 administration reduced nociceptive responses (rubbing with the ipsilateral fore- or hind-paw) in the mouse formalin orofacial pain model (5 % formalin, 10 μL subcutaneously injected into the right upper lip) [125]. BD-1047 also reduced the number of Fos-immunoreactive cells and p-p38 MAPK in the ipsilateral TNC, whereas the number of immunoreactive p-ERK cells was not modified. Using the same model, Yoon et al. demonstrated that the co-administration of clonidine with BD-1047 enhanced low-dose clonidine-induced antinociceptive effects without the sedation and hypotension side effects typically found after the administration of clonidine alone at analgesic doses. Interestingly, co-localization for α_{2A} adrenoceptors and σ_1R receptors was demonstrated in trigeminal ganglion cells [97].

8.3.5.3 Ischemic Pain

The contribution of peripheral σ_1R to ischemic pain has been recently demonstrated in a rat model of hindlimb thrombus-induced mechanical allodynia. σ_1R expression significantly increased in skin, sciatic nerve and DRG at 3 days post thrombus-induced ischemic pain in rats. Authors suggested a facilitatory effect of σ_1R on acid-sensing ion channels (ASICs) and purinergic P_2X receptors, as intraplantar injection of the σ_1R antagonist BD-1047 reduced mechanical allodynia synergistically with the ASIC blocker amiloride and the P_2X antagonist TNP-ATP [42].

8.3.5.4 Postoperative Pain

Gris et al. [126] compared the time course for thermal hyperalgesia and mechanical allodynia induced by paw incision in WT and σ_1R KO mice. No differences were found in the acquisition of thermal hyperalgesia, but σ_1R KO mice showed a faster recovery of mechanical sensitivity back to normal thresholds. c-Fos immunoreactivity was induced in the ipsilateral dorsal horn

of the spinal cord in WT mice and it was attenuated in the σ_1R KO mice 4 h after surgery. The administration of morphine and the σ_1R antagonist E-52862 4 h after surgery produced a dose-dependent antinociceptive effect, whereas ibuprofen and celecoxib were ineffective. E-52862 showed no effect in σ_1R KO mice, thus confirming the involvement of σ_1R in E-52862-mediated effects. Thus, the σ_1R seems to be involved in the sensitization to noxious stimulus induced by surgery in mice, pointing at the potential use of selective σ_1R antagonists to alleviate postoperative pain.

8.4 Concluding Remarks

The effects reported with σ_1R ligands (pronociceptive in the case of agonists and antinociceptive in the case of antagonists) are consistent with a role for σ_1R in central sensitization and pain hypersensitivity and suggest a potential therapeutic use of σ_1R antagonists for the management of neuropathic pain and other pain conditions including inflammatory, visceral, ischemic, postoperative and orofacial pain. The σ_1R seems to be devoid of its own specific signaling machinery, but it acts as a modulator of the intracellular signaling incurred upon activation of several receptors, enzymes, and ion channels relevant in pain transmission and processing. Ligands acting on σ_1R can amplify or reduce the signaling initiated when the target protein the σ_1R is interacting with becomes activated, but they are *per se* inactive. On this basis, σ_1R ligands have been postulated as ideal therapeutic drugs, effective only under pathological conditions, but inactive in normal resting/healthy conditions. Thus, while having no effects by themselves under normal physiological conditions, σ_1R ligands exert their modulatory activity under conditions involving a disturbance, such as chronic pain. This concept is very important in terms of safety and tolerability, as an ideal analgesic drug should be able to modify the stressed/dysfunctional pathway without affecting normal physiological functions. In the case of σ_1R antagonists, no adverse events have been described in rodents at doses exerting antinociceptive effects based on preclinical studies. Unlike other analgesics (e.g., opioids), σ_1R antagonists do not modify the normal sensory perception, and normal/baseline nociceptive thresholds are not modified when σ_1R antagonists are administered to normal animals. Only when the system is sensitized and hypersensitivity (i.e., allodynia and hyperalgesia) occurs following prolonged noxious stimulation (e.g., capsaicin or formalin injection) or persistent abnormal afferent input (e.g., nerve injury or inflammation) does the σ_1R antagonist exert its effect: reversion of the diminished pain thresholds back to normal sensitivity thresholds. Accordingly, σ_1R antagonists are not strictly analgesics; they are antiallodynic and antihyperalgesic drugs. Moreover, there is plenty of data supporting the combination of σ_1R antagonists with opioid therapy, which may result in a potentiation of opioid analgesia without significant increase of unwanted effects. This would result in using lower doses of opioids, with less side effects but efficacious based on the enhancement of the analgesic effect if σ_1R antagonists are used as opioid adjuvants.

Overall, based on preclinical data, the use of selective σ_1R antagonists could represent a promising efficacious and safe strategy to approach difficult-to-treat chronic pain conditions including neuropathic pain, and to enhance (or maintain) analgesic efficacy and increase the safety margin of opioids. In this regard, the most advanced investigational σ_1R antagonist, E-52862 showed a good safety, tolerability and pharmacokinetic profile in phase I studies [84]. The outcome of clinical studies with E-52862 will be of great interest to ascertain the potential of this new therapeutic approach to pain management.

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Sigma-1 Receptor Antagonists: A New Class of Neuromodulatory Analgesics

9

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Abstract

The sigma-1 receptor is a unique ligand-operated chaperone present in key areas for pain control, in both the peripheral and central nervous system. Sigma-1 receptors interact with a variety of protein targets to modify their function. These targets include several G-protein-coupled receptors such as the μ-opioid receptor, and ion channels such as the *N*-methyl-*D*-aspartate receptor (NMDAR). Sigma-1 antagonists modify the chaperoning activity of sigma-1 receptor by increasing opioid signaling and decreasing NMDAR responses, consequently enhancing opioid antinociception and decreasing the sensory hypersensitivity that characterizes pathological pain conditions. However, the participation in pain relief of other protein partners of sigma-1 receptors in addition to opioid receptors and NMDARs cannot be ruled out. The enhanced opioid antinociception

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by sigma-1 antagonism is not accompanied by an increase in opioid side effects, including tolerance, dependence or constipation, so the use of sigma-1 antagonists may increase the therapeutic index of opioids. Furthermore, sigma-1 antagonists (in the absence of opioids) have been shown to exert antinociceptive effects in preclinical models of neuropathic pain induced by nerve trauma or chemical injury (the antineoplastic paclitaxel), and more recently in inflammatory and ischemic pain. Although most studies attributed the analgesic properties of sigma-1 antagonists to their central actions, it is now known that peripheral sigma-1 receptors also participate in their effects. Overwhelming preclinical evidence of the role of sigma-1 receptors in pain has led to the development of the first selective sigma-1 antagonist with an intended indication for pain treatment, which is currently in Phase II clinical trials.

Keywords
Sigma-1 Receptors • Opioid drugs • Opioid side effects • Hyperalgesia • Allodynia • Neuropathic pain • Inflammatory pain

9.1 Introduction

Pain affects approximately 20% of the adult population, with many patients suffering from unrelieved or undertreated pain. Currently available medications (including opioids) show limited efficacy or a range of side effects (or both) that limit their use. Adequate pain management is therefore a major clinical priority (e.g. [1]), hence the importance of identifying new pharmacological targets to obtain new therapies or improve existing treatments. In this regard, as we will described in detail in this chapter, the recent appearance of overwhelming preclinical evidence for the role of sigma-1 receptors in pain has made these receptors a promising pharmacological target for the development of novel analgesics based on a groundbreaking mechanism of action.

Sigma receptors were initially misclassified as a subtype of opioid receptors in the mid-1970s [2]. It is now known that in contrast to the seven transmembrane domains of opioid receptors, the sigma-1 receptor is a single polypeptide composed by 223 amino acids, with only two transmembrane domains and no homology to opioid receptors or to any other known mammalian protein (reviewed in [3, 4]).

This intriguing receptor is expressed in anatomical areas important in pain control within the central and the peripheral nervous system. In the central nervous system, sigma-1 receptors are located in both the spinal cord (in the superficial layers of the dorsal horn) and supraspinal sites (i.e. periaqueductal gray matter, locus coeruleus and rostroventral medulla) [5–7]. In the peripheral nervous system these receptors are located in the dorsal root ganglion (DRG) [7], specifically in the soma of peripheral sensory neurons [8], and along the nerve [9] in Schwann cells [10]. The pharmacology of sigma-1 receptors is very rich and is currently well known. Sigma-1 receptors do not bind prototypic opioid drugs [7] but do bind, with high affinity, to a broad catalogue of compounds in very different structural classes and with different therapeutic and pharmacological applications, such as antipsychotics (e.g. haloperidol), antitussives (e.g. carbetapentane) and antidepressants (e.g. fluvoxamine), among many others (see [3, 4] for references). Sigma-1 receptors can also bind naturally-occurring compounds such as neurosteroids (e.g. pregnenolone, dehydroepiandrosterone, progesterone, allopregnanolone and their sulfate esters) [3, 4]. In addition, some selective high-affinity sigma-1 drugs have been used to study sigma-1 receptor function. These include the

prototypical sigma-1 agonists (+)-pentazocine and PRE-084, and the sigma-1 antagonists BD-1047, NE-100, BD-1063 (reviewed in [3]), and more recently the sigma-1 antagonist S1RA, which has been shown to exhibit exquisite selectivity for sigma-1 receptors and to lack affinity for 170 additional targets [11]. This latter compound has successfully completed Phase I clinical trials, showing good safety and tolerability [12] and it is the first sigma-1 ligand developed with an intended indication for pain treatment. S1RA is currently being assayed in Phase II clinical trials for neuropathic pain treatment and as an adjuvant to opioid analgesia [13], in light of a substantial body of preclinical evidence that supports these indications. In addition, recent studies have investigated the role of these receptors in other types of pain. In this chapter we will summarize the most important findings on the role of sigma-1 receptors in preclinical pain models.

9.1.1 Sigma-1 Receptor as a Calcium-Sensing and Ligand-Operated Chaperone: Role in Neurotransmission

At the subcellular level the sigma-1 receptor is localized in biological membranes, including microsomal, mitochondrial, nuclear and plasma membranes [14]. These receptors are particularly enriched in mitochondrion-associated endoplasmic reticulum (ER) membranes (MAM) [15]. In stress situations, the sigma-1 receptor is translocated to the ER reticular network to bind unstable IP_3 (inositol 1,4,5-trisphosphate) receptors [15] and thereby decrease their degradation, with the consequent enhancement in Ca^{2+} signaling from the ER to the mitochondria. Sigma-1 receptors then translocate to other areas of the cell, such as the plasmalemmal area within the extended ER reticular network, or to the plasma membrane itself [4], where they can physically interact with different membrane targets, thereby acting as a regulatory subunit [4, 16]. The interaction between sigma-1 receptors and their protein targets is also Ca^{2+}-dependant [17], so sigma-1 receptors act as an intracellular Ca^{2+} sensor to modulate cell physiology.

The membrane targets of sigma-1 receptors include several ion channels and G-protein-coupled receptors (GPCR). The ion channels known to interact with sigma-1 receptors are NMDA receptors (NMDARs) [17, 18], voltage-dependent K^+ channels (Kv1.2, Kv1.3, Kv1.4 and Kv1.5) [16, 19], L-type voltage-dependent Ca^{2+} channels (VDCC) [20] but not other types of VDCC [21], acid-sensing ion channels of the 1a subtype (ASIC1a) and $GABA_A$ receptors (reviewed in [4, 16]). The GPCRs known to be targeted by sigma-1 receptors are μ-opioid receptors [17, 18, 22], dopamine D_1 and D_2 receptors [17], cannabinoid receptor 1 (CB1R) [23], and serotonin receptors 1A and 2A [17]. The main protein targets of sigma-1 receptors are shown in Fig. 9.1. Interestingly, some of these proteins not only associate to sigma-1 receptors, but can also associate between themselves and act coordinately. For example, NMDARs, μ-opioid receptors and sigma-1 receptors can form a macromolecular complex to regulate the function of μ-opioid receptors (as we will describe in more detail in Sect. 2.5).

Our current understanding of sigma-1 receptor functioning assumes that the receptors are functionally inactive until the cell is stressed, when they translocate and impact the functioning of many different targets. All sigma-1 receptor client proteins noted above have been extensively shown to participate in pain neurotransmission [24–31]. Consequently, the effects of sigma-1 drugs on pain are probably complex, and are likely to take place through the simultaneous modulation of numerous intracellular pathways.

Sigma-1 agonists mimic the stress response, with the consequent translocation of sigma-1 receptors, whereas sigma-1 antagonists prevent this process [15]. The binding of agonists or antagonists to sigma-1 receptor induces distinct conformational changes in the receptor: sigma-1 agonists increase the separation between the *N*- and *C*-termini, whereas sigma-1 antagonists have the opposite effect [32]. These conformational changes in response to ligands are expected to alter the ability of the sigma-1 receptor to interact with its protein targets, and

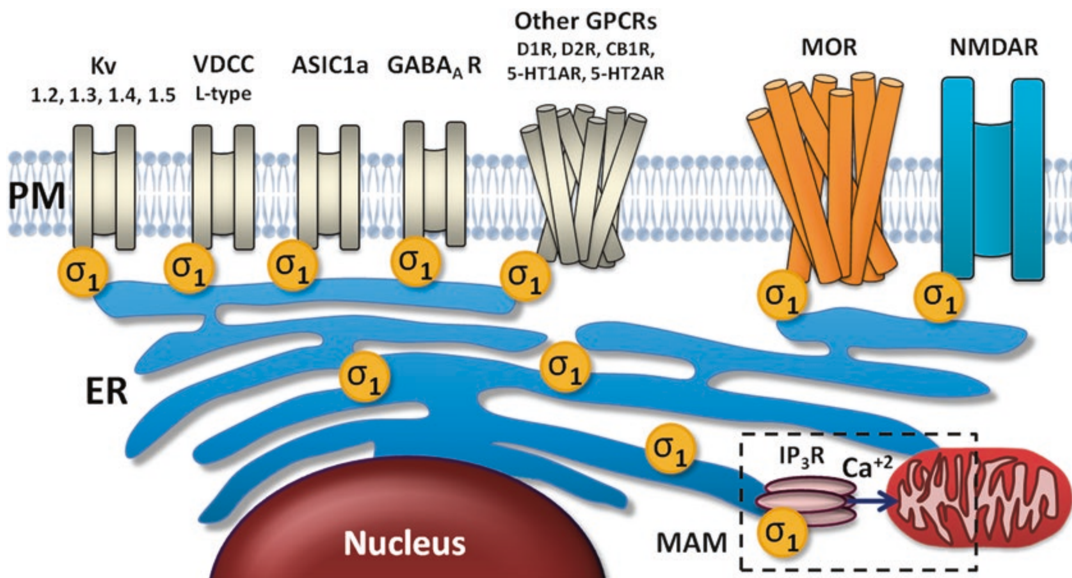

Fig. 9.1 Main protein targets of sigma-1 receptors (σ_1). Sigma-1 receptors located in the mitochondrion-associated ER (endoplasmic reticulum) membrane (MAM) bind to inositol 1,4,5-trisphosphate receptors (IP_3R), potentiating the influx of Ca^{2+} from the ER into the mitochondria. In addition, sigma-1 receptors located in the ER reticular network can, through protein–protein interactions, modulate several receptors and channels expressed in the plasma membrane (PM). The two best known protein targets of sigma-1 receptors are the *N*-methyl-*D*-aspartate receptor (NMDAR) and the µ-opioid receptor (MOR), shown here larger than scale for clarity. Other client proteins of sigma-1 receptors at the PM include voltage-gated K^+ channels (Kv1.2, 1.3, 1.4, 1.5), L-type voltage-dependent calcium channels (VDCC), acid-sensing ion channels of the 1a subtype (ASIC1a), $GABA_A$ receptors ($GABA_AR$), and other G-protein-coupled receptors (GPCRs) such as D1R and D2R, CB1R, 5-HT1AR and 5-HT2AR (see text for details)

consequently to influence the neuromodulatory effects of this receptor.

9.2 The Anti-opioid Sigma-1 System: Modulation of Opioid-Induced Analgesia and Side Effects

Opioid drugs, especially µ-agonists (such as morphine, codeine, fentanyl, oxycodone, buprenorphine or tramadol), are widely used in clinical practice to treat moderate to severe pain [33]. Unfortunately, in addition to analgesia, these drugs produce a myriad of other effects in human patients, including myosis, nausea, mental confusion, respiratory depression and constipation as well as tolerance, withdrawal and dependence. Some of these nonanalgesic effects constitute worrisome opioid side effects which are highly relevant in clinical practice because they may limit the use of opioids (reviewed in [33]).

In contrast to opioids, and in spite of the location of sigma-1 receptors along pain pathways, sigma-1 antagonists are unable to relieve pain per se in nonsensitized conditions (nociceptive pain) in response to either thermal (e.g. [34–36]) or mechanical stimuli (e.g. [36–39]). However, as we will describe below, there is currently a growing body of preclinical evidence that supports the use of sigma-1 antagonists to increase the therapeutic index (ratio of analgesic efficacy vs side effects) of opioids.

9.2.1 Potentiation of Opioid Antinociception by Systemic Sigma-1 Inhibition

The first evidence of the role of sigma-1 receptors in pain appeared in the early to mid-1990s. Chien

and Pasternak studied the effects on morphine antinociception of haloperidol, a compound that at the time was considered a prototypical sigma antagonist because no other more selective drugs were available. They found that the systemic administration of haloperidol, although lacking analgesic activity alone, enhanced morphine antinociception to noxious heat stimulation (tail-flick test) [34, 40, 41]. These studies were the first to suggest the possibility of a tonically active anti-opioid sigma-1 system, and formed the basis for subsequent studies. Subsequent studies that investigated the systemic administration of opioids and sigma ligands are described below and summarized in Table 9.1.

The enhancement of morphine analgesia to heat stimuli initially described for the nonselective sigma-1 antagonist haloperidol was more recently replicated with other sigma-1 antagonists including the highly selective S1RA, and the effect was also found for mechanical stimulation [7, 39, 42]. In addition, pharmacological sigma-1 antagonism also increased the antinociceptive effect (to either thermal or mechanical stimuli) induced by the systemic administration of other clinically relevant μ-opioids in addition to morphine, including fentanyl, oxycodone, codeine, buprenorphine and tramadol [7, 39, 42]. See Table 9.1 for details.

The modulation of μ-opioid antinociception by sigma-1 receptors is not restricted to this opioid receptor subtype, as shown by reports that sigma-1 antagonism also potentiated the effects induced by the κ-opioid agonist U50,488H [34, 41, 43, 44], the κ_3-opioid agonist naloxone benzoylhydrazone (NalBzoH) [34, 41] and the nonselective κ-opioid analgesic (−)-pentazocine [45]. Among studies of the systemic administration of combinations of sigma-1 antagonists and opioid agonists, only one discordant result has appeared to date, in which the sigma-1 antagonist BD-1047 unexpectedly produced a decrease in U50,488H-induced antinociception [46]. Although BD-1047 is widely used as a sigma-1 antagonist, its selectivity has been tested in a panel of only 10 receptors [47]. Therefore, a possible explanation for the discordant effects induced by BD-1047 is that this drug might interact with one or more unknown targets and thus interfere with opioid antinociception. See Table 9.1 for details of the different combinations of κ-opioids and sigma-1 ligands tested as of this writing.

Further experiments were recently carried out with sigma-1 receptor knockout (KO) mice. The mutant animals showed a marked increase in antinociception induced by several μ-opioid agonists to a mechanical stimulus [7, 39], but showed no increase in opioid antinociception to thermal stimulation [42]. See Table 9.1 for details of the opioids tested in sigma-1 KO mice and the types of stimulation used. Interestingly, the sigma-1 antagonist S1RA, which markedly enhanced opioid-induced thermal antinociception in wild-type mice, was unable to do so in sigma-1 KO mice [42]. This finding supports the involvement of on-target mechanisms in the effect of this drug. Studies of pain mechanisms that used pharmacological and genetic inhibition of other receptors have reported conflicting results (e.g. [48, 49]), which have been attributed to the development of compensatory mechanisms in mutant animals. However, it is interesting that these purported compensatory mechanisms are seen in models of opioid-induced thermal antinociception but not in mechanical antinociception. The pain pathways of mechanical and thermal stimulation do not completely overlap [50], and the neurochemical mechanisms underlying opioid-induced mechanical and thermal antinociception differ (e.g. [51–53]). These differences might therefore differentially affect the development of compensatory mechanisms in sigma-1 KO animals. Although the pharmacological antagonism of sigma-1 receptors is able to enhance opioid antinociception to both mechanical and thermal stimulation, a hypothesis that warrants further study is that there are differences on the mechanism of action of sigma-1 receptors in these processes.

9.2.2 Opioid Antinociception and Sigma-1 Inhibition: A Central or Peripheral Interaction?

Both opioid and sigma-1 receptors are expressed throughout the nociceptive neural circuitry at both

Table 9.1 Summary of the effects of systemic subcutaneous or intraperitoneal treatment with combinations of opioids and sigma-1 antagonists in wild-type animals, and the effects of systemic opioids in sigma-1 knockout (KO) mice

Opioid receptor subtype	Opioid drug	Sigma-1 antagonist/ KO	Effect on opioid antinociception	Stimulus	References
μ	Morphine	Haloperidol	Enhancement	Heat	[34, 40, 41]
		S1RA			[42]
		S1RA	Enhancement	Mechanical	[7, 39]
		BD-1063			
		BD-1047			[39]
		NE-100			
		KO	No effect[a]	Heat	[42]
			Enhancement	Mechanical	[7, 39]
	Fentanyl	S1RA	Enhancement	Heat	[42]
		S1RA	Enhancement	Mechanical	[7]
		BD-1063			
		KO	No effect[a]	Heat	[42]
			Enhancement	Mechanical	[7]
	Oxycodone	S1RA	Enhancement	Heat	[42]
		BD-1063	Enhancement	Mechanical	[7]
		KO	Enhancement	Mechanical	[7]
	Codeine	S1RA	Enhancement	Heat	[42]
	Buprenorphine	S1RA	Enhancement	Heat	[42]
		BD-1063	Enhancement	Mechanical	[7]
		KO	No effect[a]	Heat	[42]
			Enhancement	Mechanical	[7]
	Tramadol	S1RA	Enhancement	Heat	[42]
		BD-1063	Enhancement	Mechanical	[7]
		KO	Enhancement	Mechanical	[7]
κ	U50,488H	Haloperidol	Enhancement	Heat	[34, 41]
		MR200			[43]
		(−)-MRV3			[44]
		BD-1047	Inhibition	Heat	[46]
	NalBzoH	Haloperidol	Enhancement	Heat	[34, 41]
	(−)-Pentazocine	Haloperidol	Enhancement	Heat	[45]

[a]Although sigma-1 KO animals did not show increased opioid-induce analgesia, sigma-1 antagonists potentiated the effects of these opioid drugs in wild-type mice but lacked this effect in mutant animals, suggesting the involvement of on-target mechanisms in the effects of the sigma-1 antagonists tested

central and peripheral levels [5–7, 33]. Therefore, several studies have been done to determine the anatomical location of the modulation of opioid antinociception by sigma-1 receptors. Opioid analgesia is known to be more prominent at central levels, particularly supraspinally in the so-called descending pathways [54]. Accordingly, most studies have focused on the central modulation of opioid analgesia by sigma-1 receptors. Several approaches have been used to this end: the combined intracerebroventricular (i.c.v.) administration of opioids and sigma-1 antagonists [17, 18, 55], specific antisense oligodeoxynucleotides (ASOs) to inhibit the expression of sigma-1 receptors [55–57], or the systemic administration of an opioid agonist and the supraspinal inhibition of sigma-1 receptors [56–58] or vice versa [59]. All these combinations led to an increase in opioid-induced antinociception to thermal stimuli. This enhanced supraspinal opioid antinociception has

been demonstrated for the gold standard μ analgesic morphine [17, 18, 55–57] and the selective μ agonist DAMGO (D-Ala[2],Me-Phe[4],Gly-ol[5]enkephalin) [59], as well as for the κ agonists U50,488H [59] and NalBzoH [56–58], and the δ agonist DPDPE ([D-Pen[2], D-Pen[5]]enkephalin) [56, 57]. Therefore, supraspinal sigma-1 receptors are able modulate opioid antinociception induced by μ, κ or δ opioid agonists. See Table 9.2 for details of the combinations of opioids and sigma-1 ligands tested thus far to study supraspinal antinociception to heat stimuli.

The specific supraspinal sites involved in the modulation of morphine-induced thermal antinociception by sigma-1 receptors have also been determined. The rostroventral medulla (RVM), the periaqueductal gray (PAG) and the locus coeruleus (LC) constitute important sites in the circuit responsible for the descending modulation of pain [26], and although sigma-1 receptors are expressed in these three areas [7, 17, 18, 60], the modulation of morphine antinociception differs depending on the injection site [55]. The administration of haloperidol or specific sigma-1 ASOs in the RVM markedly enhanced the antinociceptive actions of coadministered morphine, implying a strong tonic activity of the sigma-1 system in this site. Although this tonic sigma-1 inhibitory activity was not observed in either the LC or the PAG (as evidenced by the lack of response to haloperidol coadministered with morphine in these regions), the pharmacological antagonism of sigma-1 receptors in the RVM by haloperidol enhanced PAG morphine analgesia [55]. These studies illustrate the pharmacological importance of sigma-1 receptors in the brainstem modulation of opioid analgesia, in particular in the RVM. See Table 9.2 for a summary of these experiments and their results.

In addition to the widely reported central modulation of opioid antinociception by sigma-1 receptors, their modulatory role on peripheral opioid antinociception was recently explored. The enhanced opioid antinociception to mechanical stimuli by the combination of the systemic administration of different μ opioid drugs (morphine, fentanyl, oxycodone, buprenorphine and tramadol) and sigma-1 antagonists was shown to be reversed by the peripherally restricted opioid antagonist naloxone methiodide [7], indicating that peripheral opioid receptor activation participates in the modulatory effect of sigma-1 antagonists. In fact, sigma-1 receptor inhibition was sufficient to unmask the strong antinociceptive effects of loperamide [7], a peripherally acting opioid analgesic used clinically as an antidiarrheal drug and not as an analgesic [61]. Furthermore, local sigma-1 peripheral antagonism was able to greatly enhance opioid antinociception induced by the systemic administration of all opioids mentioned above [7], and the local peripheral coadministration of morphine with sigma-1 antagonists was even able to induce strong opioid antinociception [39]. Further support for the peripheral antinociceptive synergism between sigma-1 inhibition and opioid agonism comes from the observation that the potentiation of opioid mechanical antinociception seen in sigma-1 KO mice was abolished by naloxone methiodide [7], and local morphine administration was able to induce much stronger antinociception in this mutant strain than in wild-type mice [39]. These results clearly show that the modulation of μ-opioid antinociception by sigma-1 receptors is not restricted to central sites, and thus constitute the first reported evidence of the role of peripheral sigma-1 receptors in pain.

In summary, it is clear that central sigma-1 inhibition enhances opioid antinociception to thermal stimuli, and that peripheral sigma-1 antagonism enhances opioid antinociception to mechanical stimuli. Taking into account the neurochemical differences noted above in mechanical and thermal opioid antinociception, it may be worth testing whether central sigma-1 inhibition is able to increase opioid antinociception to mechanical stimuli, and whether peripheral sigma-1 antagonism enhances opioid antinociception to thermal stimuli. The results of these studies would provide a more complete picture of the effects of sigma-1 inhibition on opioid antinociception. Although there are undoubtedly still some gaps in this field of research, it is well established that sigma-1 antagonism enhances opioid antinociception by acting on several steps in the pain circuitry.

NalBzoH Naloxone benzoylhydrazone, *DPDPE* [D-Pen[2], D-Pen[5]]enkephalin, *DAMGO* (D-Ala[2],Me-Phe[4],Gly-ol[5]enkephalin), *s.c.* subcutaneous, *i.c.v.* intracerebroventricular, *i.t.* intra-

Table 9.2 Summary of studies designed to elucidate the central and peripheral modulation of opioid antinociception in wild-type animals treated with sigma-1 antagonists or ASOs, and in sigma-1 KO mice

Opioid receptor subtype	Opioid drug	Route	Sigma-1 antagonist/ ASO/KO	Route	Effect on opioid antinociception	Stimulus	References
μ	Morphine	i.c.v.	S1RA	i.c.v.	Enhancement	Heat	[18]
			BD-1047				
			BD-1063				
			NE-100				
		i.c.v.	ASO	i.c.v	Enhancement	Heat	[56, 57]
		RVM	Haloperidol	RVM	Enhancement	Heat	[55]
		PAG		PAG	No effect		
				RVM	Enhancement		
		LC		LC	No effect		
		RVM	ASO	RVM	Enhancement	Heat	
		PAG					
		s.c.	BD-1063	i.pl.	Enhancement	Mechanical	[7]
		i.pl.	BD-1063	i.pl.	Enhancement	Mechanical	[39]
			BD-1047				
			NE-100				
			S1RA				
			KO	-			
	DAMGO	i.c.v	(+)-MR200	s.c.	Enhancement	Heat	[59]
	Fentanyl	s.c.	BD-1063	i.pl.	Enhancement	Mechanical	[7]
			S1RA				
	Oxycodone	s.c.	BD-1063	i.pl.	Enhancement	Mechanical	[7]
	Buprenorphine	s.c.	BD-1063	i.pl.	Enhancement	Mechanical	[7]
	Tramadol	s.c.	BD-1063	i.pl.	Enhancement	Mechanical	[7]
	Loperamide	s.c.	BD-1063	s.c.	Enhancement	Mechanical	[7]
				i.pl.			
			S1RA	s.c.			
				i.pl.			
κ	U50,488H	s.c.	ASO	i.c.v.	Enhancement	Heat	[56, 58]
		i.c.v.	(+)-MR200	s.c.	Enhancement	Heat	[59]
	NalBzoH	s.c.	ASO	i.c.v	Enhancement	Heat	[56–58]
δ	DPDPE	i.c.v.	ASO	i.c.v.	Enhancement	Heat	[56, 57]
			(+)-MR200	s.c.	Enhancement	Heat	[59]
		i.t.	Haloperidol	s.c.	Enhancement	Heat	[34]

thecal, *RVM* rostroventral medulla, *PAG* periaqueductal gray, *LC* locus coeruleus, *ASO* antisense oligodeoxynucleotide, *KO* knockout.

9.2.3 Sigma-1 Agonism and Opioid Antinociception

In contrast to the enhanced opioid antinociception induced by sigma-1 antagonists, the systemic administration of the selective sigma-1 agonist (+)-pentazocine decreased the antinociception to heat stimuli induced by morphine, U50,488H, NalBzoH, DPDPE, and even antinociception by its levorotatory isomer, the κ-opioid (−)-pentazocine [34, 40, 43, 46, 55, 57, 62]. This observation is interesting because the racemic mixture of pentazocine is used as an analgesic in humans [63]. Therefore, the (+)-pentazocine from this mixture, through sigma-1 agonism, may interfere with the opioid analgesia induced by (−)-pentazocine [45, 62]. However, the relevance for

clinical practice of the interaction between pentazocine and sigma-1 receptors is still unknown.

Interestingly, the prototypic sigma-1 agonist PRE-084, unlike (+)-pentazocine, was unable to decrease the antinociceptive effect of morphine to either thermal [18] or mechanical stimuli [39], or to diminish the effect of fentanyl on mechanical nociception (unpublished data). However, PRE-084 clearly reversed the effects of sigma-1 antagonists on opioid antinociception [18, 39] and in several additional pain situations [36–38], clearly indicating that this drug acts through sigma-1 receptors. At this time it is unclear why (+)-pentazocine and PRE-084 exert different effects on opioid antinociception. However, it was recently reported that the in vivo i.c.v. administration of recombinant sigma-1 receptor to sigma-1 KO mice was sufficient to restore sigma-1 receptor function and decrease morphine antinociception [18], indicating that recovery (with a nonpharmacological approach) of function in these receptors is able to decrease opioid antinociception.

9.2.4 Modulation of Opioid Side Effects by Sigma-1 Receptors

Clinically, a dose-limiting factor in obtaining maximal analgesia with opioids is the risk of adverse side effects. The obvious importance of studying how sigma-1 receptors modulate the nonalgesic effects of opioids lies in the assumption that if they modify both analgesic and nonalgesic effects of opioids equally, this would limit their potential use for the clinical development of sigma-1 antagonists as opioid adjuvants to enhance opioid analgesia. Consequently, the role of sigma-1 receptors in modulating the nonanalgesic effects of opioids has been explored in laboratory animals.

Opioids can have paradoxical effects in rodents: although they produce myosis and sedation in humans [33], they induce mydriasis [42] and hyperlocomotion [39] in rodents. The administration of S1RA alone in rodents did not modify pupil diameter, and when associated with morphine, it did not modify the mydriasis induced by this opioid [42]. In addition, morphine-induced hyperlocomotion was not modified by sigma-1 inhibition in sigma-1 KO mice [39]. Because these two central nonanalgesic effects of opioids do not correlate with symptoms in human patients who use opioids, the translatability of these findings is limited, although they clearly show that sigma-1 receptors do not modulate all opioid effects indistinctly.

The role of sigma-1 receptors on other more clinically relevant opioid effects with a strong central component has also been explored. Sigma-1 pharmacological antagonism (by S1RA) did not change the severity of somatic manifestations of naloxone-induced morphine withdrawal or the development of morphine tolerance in mice [42], which are highly relevant opioid side effects in humans [33]. In fact, sigma-1 antagonism was even able to restore morphine antinociception in morphine-tolerant mice, and interestingly, the rewarding effects of morphine (evaluated as place conditioning) were antagonized [42]. This latter result, although it goes against the widely reported enhancement of opioid antinociception by sigma-1 antagonism, is consistent with other preclinical studies that proposed the use of sigma-1 antagonists as promising tools for the treatment of addiction and dependence induced by other drugs of abuse (e.g. [64]). In addition, the sigma-1 agonist (+)-pentazocine did not affect morphine-induced lethality, suggesting that it did not affect the respiratory depression induced by this opioid [34], which is one of the most worrisome effects of opioid use [33].

In addition to primarily central nonanalgesic opioid effects, the role of sigma-1 receptors has been evaluated in gastrointestinal transit inhibition, which is produced mainly peripherally [33]. This opioid effect is of clinical relevance since is one of the main reasons for patients' voluntary withdrawal from opioid medication [65]. The sigma-1 agonist (+)-pentazocine was unable to modify the morphine-induced decrease in gastrointestinal transit [34], and sigma-1 inhibition (by sigma-1 antagonists or in sigma-1 KO mice) did not alter the effects on gastrointestinal transit

Table 9.3 Summary of the nonanalgesic effects of opioids in wild-type animals treated with sigma-1 antagonists and in sigma-1 KO mice. All opioid drugs and sigma-1 antagonists were administered systemically

Opioid side effect	Opioid	Sigma-1antagonist/KO	Effect	References
Mydriasis	Morphine	S1RA	No effect	[42]
Increased locomotor activity	Morphine	KO	No effect	[39]
Gastrointestinal transit inhibition	Morphine	KO	No effect	[39]
	Fentanyl	BD-1063	No effect	[7]
		KO		
	Loperamide	BD-1063	No effect	[7]
		KO		
Dependence	Morphine	S1RA	Decreased	[42]
Tolerance	Morphine	S1RA	Decreased	[42]

KO knockout

induced by morphine, fentanyl or the peripherally-restricted opioid loperamide.

Experiments that studied the effects of sigma-1 inhibition on nonanalgesic effects of opioids are summarized in Table 9.3.

The findings summarized above show that sigma-1 receptors do not appear to modulate either centrally- or peripherally-induced nonanalgesic effects of opioids. Regardless of the exact mechanistic nature of the differential modulation of opioid antinociception and adverse events by sigma-1 receptors, these findings point to a potentially beneficial avenue of research aimed at improving the safety profile of opioid drugs.

9.2.5 The Molecular Mechanism of the Modulation of Opioid Antinociception by Sigma-1 Receptors: Releasing the Brake on Opioid Analgesia

The interaction between NMDAR activity and opioid antinociception and tolerance was reported in the early 1990s, when NMDAR antagonists were found to increase opioid functioning. It is now known that μ-opioid receptors and NMDARs establish physical interactions (reviewed in [66]). Together with this complex, the μ-opioid receptor carries the histidine triad nucleotide-binding protein 1 (HINT1). This latter protein plays a role in a series of events that occur after μ-opioid receptor agonism, leading to the activation of protein kinase C γ (PKCγ), which phosphorylates NMDARs at the NR1 subunit. This phosphorylation of NMDARs releases them from the μ-opioid receptor–HINT1 complex, and thereby enhances NMDAR activity that in turn promotes the permeation of Ca2+ ions into the cytosol. Increased Ca2+ levels activate Ca2+–calmodulin (CaM) complex to enhance the activity of calmodulin-dependent kinase II (CaMKII), which in turn acts on the μ-opioid receptor decreasing its activity (reviewed in [66, 67]. In parallel, another result of increased intracellular Ca2+ is enhanced binding of the Ca2+–CaM complex to NMDARs to decrease their activity. This latter event constitutes a Ca2+-dependent feedback mechanism that inhibits NMDARs and thus prevents excessive Ca2+ entry in the cytosol [68].

In this complex process, sigma-1 receptors play a pivotal role in the functional interaction between the μ-opioid receptor and NMDA. The sigma-1 receptor is located in the complex formed by the μ-opioid receptor, HINT1 and NMDAR. When NMDARs are active, increased Ca2+ influx induces the binding of sigma-1 receptors to NMDARs, which protects the latter from the inhibitory effect of Ca2+–CaM. This loss of inhibition plays an important role in the enhanced activity of NMDARs, and consequently in decreased μ-opioid signaling [17, 18]. See Fig. 9.2a, b.

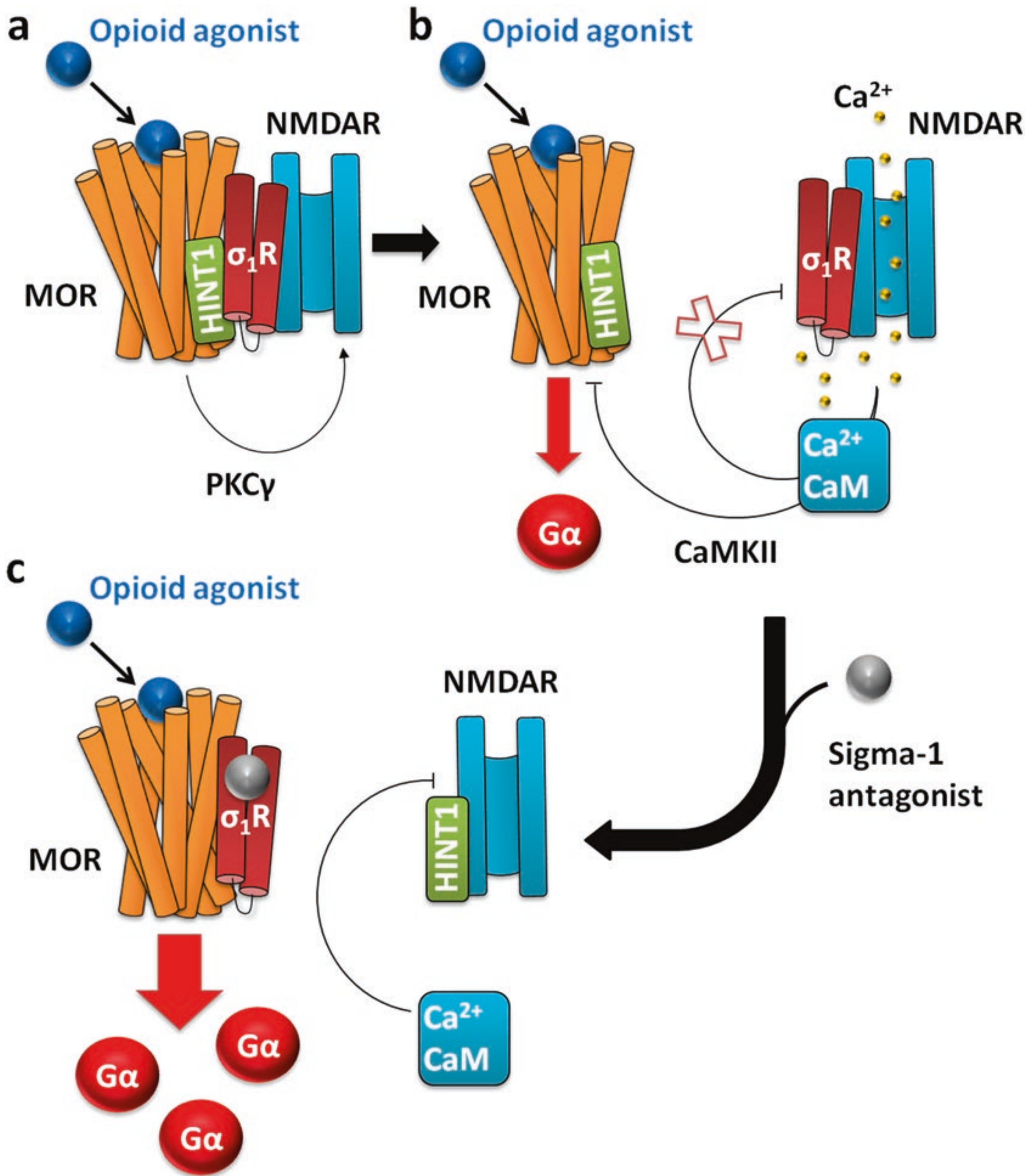

Fig. 9.2 Schematic representation of the interaction between sigma-1 receptor (σ_1R), μ-opioid receptor (MOR), NMDA receptor (NMDAR) and histidine triad nucleotide-binding protein 1 (HINT1). **(a)** MOR activation by an agonist leads to the activation of NMDARs through protein kinase C γ (PCKγ). **(b)** NMDAR activation promotes Ca^{2+} influx and facilitates the interaction of σ_1R with NMDARs, which impedes the inhibitory action of Ca^{2+}–calmodulin (CaM) on NMDARs. Ca^{2+}–CaM impacts negatively on MOR signaling by the activation of calmodulin-dependent kinase II (CaMKII). **(c)** Sigma-1 antagonists stabilize the association of σ_1R to MOR, and the transfer of HINT1 to NMDARs. When NMDARs do not bind σ_1Rs, they are more susceptible to the inhibitory effect of CaM. As a result their inhibitory influence on MOR signaling is reduced. See text for details

When the sigma-1 receptor is absent from the μ-opioid receptor–HINT1 complex (as shown in Fig. 9.2b), this complex is able to interact with other sigma-1 receptors and recruit the activity of additional NMDARs, thereby amplifying the inhibitory effect on opioid functioning. Sigma-1 agonists facilitate the transfer of sigma-1 receptors to NMDARs, increasing the speed of this process [17]. In addition, sigma-1 antagonists maintain the binding of sigma-1 receptors to μ-opioid receptors, and facilitate the transfer of HINT1 to NMDARs. In this situation the NMDARs lack the protection provided by sigma-1 receptors against the binding of Ca2+–CaM, which can then easily interact with NMDARs and curtail their activity [17, 18] (see Fig. 9.2c). Therefore, the enhanced opioid signaling by sigma-1 antagonists [22] is a consequence of decreased NMDAR activity. In other words, sigma-1 receptors facilitate the action of NMDA in blocking the function of opioids, whereas sigma-1 antagonists disrupt the action of this biological brake on opioid signaling, with the resulting enhancement of opioid analgesia and decrease in (or even rescue from) opioid tolerance [17, 18].

The differential impact of sigma-1 receptors on the modulation of μ-opioid analgesia and side effects suggests that the complex formed by μ-opioid receptors, NMDAR and sigma-1 receptors might be present in specific subsets of neurons involved in pain pathways but not in other processes. Further studies are needed to understand the complexities of the effects of sigma-1 on opioid modulation.

The direct coupling between sigma-1 and other opioid receptor subtypes has not yet been demonstrated, although it is a likely phenomenon since sigma-1 inhibition is widely reported to modulate the effects and signaling mediated by κ- and δ-opioids (as reviewed in the preceding sections). It is worth noting that in addition to opioid receptors, several protein targets of sigma-1 receptors are known to participate in opioid effects. For example, L-type Ca2+ channels are among the downstream effectors of opioid signaling [33]. Therefore, the behavioral impact of the modulation of opioid effects by sigma-1 receptors might be the result of simultaneous complex interactions between several membrane targets of sigma-1 receptors, and not an exclusive result of the direct modulation of opioid–NMDA receptors.

9.3 Pain Modulation by Sigma-1 Receptors in the Absence of Opioid Drugs: Effects on Animal Models of Tonic and Chronic Pain

In addition to the role of sigma-1 receptors in modulating the effects of opioids on acute nociceptive pain, it has been widely reported that sigma-1 inhibition ameliorates other types of pain without the concomitant administration of any opioid drug. A large part of our knowledge about the analgesic potential of sigma-1 receptors is based on findings in chemically-induced pain, but the most conclusive findings have been obtained in more clinically relevant models of pathological pain.

9.3.1 Sigma-1 Receptors and Pain Induced by Chemical Irritants: Formalin and Capsaicin

Formalin is one of the chemical irritants used most widely in pain research [69]. This irritant induces a biphasic pain response in rodents: an initial acute pain response (first phase) due to the direct activation of nociceptors, followed by a prolonged tonic response (second phase) characterized by spontaneous activity of primary afferent neurons together with functional changes in the spinal cord (central sensitization) and accompanied by an edematous process [69]. Although in pain studies formalin is classically administered into the paw, it can also be injected into the trigeminal area (e.g. [70]) to study orofacial pain, which is a distinct type of pain known to be resistant to most analgesics [71].

The first reported evidence for the role of sigma-1 receptors in a model of chemically-induced pain was obtained with the formalin test. Sigma-1 KO mice showed a significant decrease

in both phases of nociception after the intraplantar injection of formalin [72]. Moreover, the systemic pharmacological antagonism of sigma-1 receptors in studies that used nonselective (e.g. haloperidol) or selective drugs (e.g. S1RA) also decreased both phases of formalin-induced pain when the chemical irritant was administered into the paw [11, 32, 35, 73–75] or into the trigeminal area [76, 77]. Although all sigma-1 antagonists tested decreased formalin-induced nociception, the second phase of formalin-induced pain was shown to be more sensitive to sigma-1 antagonism than the first phase: lower doses of sigma-1 antagonists were able to reduce second-phase pain [35, 75]. Some systemic treatments (e.g. haloperidol metabolite I) reduced only the second phase of formalin-induced nociception without altering the first phase [35] (see Table 9.4 for details). Since the altered processing of sensory information at central levels is involved in the second phase of formalin-induced pain, further studies were conducted to determine whether central sigma-1 antagonism could ameliorate formalin-induced pain. It was found that the intrathecal (i.t.) or i.c.v. administration of sigma-1 antagonists was effective in reducing the pain induced by this chemical irritant [75, 78]. Importantly, the effects of sigma-1 antagonists were accompanied by a reduction in the phosphorylation (activation) of key proteins involved in central sensitization [76, 78], including the NR1 subunit of NMDAR [78]. In addition, sigma-1 antagonism was able to increase noradrenalin levels in the spinal cord (while decreasing glutamate release), and the effect of sigma-1 antagonism on formalin-induced nociception was reversed by α_2-adrenoceptor antagonism, suggesting the involvement of descending inhibitory pathways in the mechanism of action of sigma-1 inhibition and decreased pain [75].

Interestingly, it was recently reported that sigma-1 antagonism at the site of administration of formalin was also able to reduce pain behavior, indicating that peripheral sigma-1 receptors also participate in the decrease in formalin-induced pain by sigma-1 inhibition [75]. These results indicate that sigma-1 receptors facilitate formalin-induced pain at central sites (both spinally and supraspinally) and also peripherally.

Further experiments were performed with capsaicin as a chemical irritant. The intradermal injection of capsaicin decreased the mechanical pain threshold in the area surrounding the injection even when the area was not stimulated with capsaicin (the so-called area of secondary hypersensitivity). This decrease in the mechanical threshold was attributed to central sensitization [79]. Exploring the effects of sigma-1 inhibition on capsaicin-induced secondary mechanical hypersensitivity is of interest because this model is widely used in clinical research to test the effects of drugs on mechanical allodynia (e.g.

Table 9.4 Summary of the effects on formalin-induced nociception of sigma-1 antagonists administered to wild-type animals and sigma-1 KO mice

Formalin	Sigma-1 antagonist/ KO	Administration	Phases: First/Second/Both	References
Paw	KO	–	Both	[72]
	Haloperidol	Systemic (s.c./i.p.)	Both	[32, 35]
	Haloperidol met II			[35]
	Haloperidol met I		Second	[35]
	S1RA		Both	[11, 74, 75]
	BD1047	i.t.	Second	[78]
	BMY-14802		Both	
	S1RA		Both	[75]
	S1RA	i.c.v.	Both	[75]
		i.pl.		
Orofacial	BD1047	i.p.	Both	[76, 77]

s.c. subcutaneous, *i.p.* intraperitoneal, *i.t.* intrathecal, *i.c.v.* intracerebroventricular, *KO* knockout

[80]), and it is considered a surrogate model of neuropathic pain, since antineuropathic drugs show antiallodynic activity in this test in both humans and rodents [37, 38, 80]. It has been shown that sigma-1 KO mice do not sensitize to mechanical stimuli in response to capsaicin [37], and this phenotype was mimicked by sigma-1 antagonists such as the nonselective drug haloperidol or selective sigma-1 antagonists including BD-1063 or S1RA [11, 37, 38, 73].

Importantly, the effects of sigma-1 inhibition on either capsaicin-induced secondary mechanical hypersensitivity or formalin-induced pain were not reversed by the opioid antagonist naloxone [35, 38, 75]. This observation indicated conclusively that these effects are independent of modulation of the opioidergic system, and that sigma-1 receptors can act through other mechanisms to decrease pain transmission.

Both formalin and capsaicin are known to be C-fiber activators, although they act through different receptors. Formalin activates TRPA1 (transient receptor potential ankyrin 1), whereas capsaicin stimulates TRPV1 (transient receptor potential vanilloid 1) [50]. The repeated activation of C fibers is known to increase the excitability of spinal cord neurons, a phenomenon named wind-up, which can contribute to the establishment of central sensitization [81, 82]. This process is known to be inhibited in sigma-1 KO mice [83] or by S1RA in wild-type animals [11, 84], and these findings support the role of sigma-1 receptors in the spinal neuron hyperexcitability that contributes to pain hypersensitivity after the injection of formalin or capsaicin.

The usefulness of capsaicin administration as a pain model is not restricted to somatic pain, since this chemical algogen can be also administered in the gut to produce visceral pain. Capsaicin-induced visceral pain shows two distinct components – intense (acute) pain and referred mechanical hyperalgesia – and these two components have been reported in humans [85] and rodents [86]. The pathophysiological mechanisms and response to drug treatments are different in somatic and visceral pain [87]. In fact, unlike somatic afferents, visceral afferents are known to be unable to trigger wind-up in the spinal cord (reviewed in [81]), hence the interest in testing the effects of sigma-1 receptor inhibition in a model of visceral pain. The systemic administration of several selective sigma-1 antagonists (BD-1063, S1RA and NE-100) attenuated both acute pain-related behaviors and referred mechanical hyperalgesia induced by intracolonic capsaicin [88]. Interestingly, sigma-1 KO mice mimicked the effects of sigma-1 antagonists in the decrease in acute pain-like behaviors induced by intracolonic capsaicin, but did not show any amelioration of referred hyperalgesia. However, sigma-1 antagonists were devoid of effect in sigma-1-KO mice, indicating that the effects of these drugs are specifically mediated by sigma-1 receptors, and that the absence of the referred hyperalgesia phenotype in sigma-1-KO mice was probably due to the development of compensatory mechanisms [88].

At the time of writing, no studies have been done to identify the anatomical location of pain modulation by sigma-1 receptors in nociceptive behaviors or sensory hypersensitivity induced by capsaicin (administered to somatic or visceral tissues). Current evidence for the role of sigma-1 receptors in central sensitization suggests that central sigma-1 receptors probably play a role in the effects seen to date. However, taking into account the combined contributions of peripheral and central sigma-1 receptors to pain induced by other chemical irritants (e.g. formalin), the participation of peripheral sigma-1 receptors in capsaicin-induced pain or sensitization cannot be ruled out, and further studies are needed to clarify this issue.

Studies that have tested the effect of sigma-1 inhibition by administering sigma-1 antagonists to wild-type or sigma-1 KO mice are summarized in Table 9.5.

9.3.2 Sigma-1 Receptors and Neuropathic Pain

Neuropathic pain is one of the most challenging types of chronic pain conditions to treat, and new therapeutic tools are clearly needed [89]. The results of work in the modulation of formalin- and

Table 9.5 Summary of the effects on capsaicin-induced pain of sigma-1 receptor inhibition by the systemic administration of sigma-1 antagonists in wild-type animals or in sigma-1 knockout mice. Mechanical allodynia and referred hyperalgesia were assessed with von Frey filaments, and acute pain behaviors were evaluated with observational methods

Capsaicin	Readout	Sigma-1 antagonist/KO	Effect	References
i.pl.	Mechanical allodynia	Haloperidol	Attenuation	[38]
		Haloperidol Metabolite I		
		Haloperidol Metabolite II		
		BD1063	Attenuation	[37]
		BD1047		
		NE100		
		S1RA	Attenuation	[11]
		KO	Attenuation	[37]
i.cl.	Acute pain	BD1063	Attenuation	[88]
		NE-100		
		S1RA		
		KO	Attenuation	
	Referred hyperalgesia	BD1063	Attenuation	
		NE-100		
		S1RA		
		KO	No effect[a]	

i.pl. intraplantar, *i.cl.* intracolonic, *KO* knockout
[a]Although sigma-1 KO animals showed referred hyperalgesia equivalent to that seen in wild-type mice, sigma-1 antagonists induced a clear antihyperalgesic effect in wild-type mice but not in mutant animals, indicating the involvement of on-target mechanisms in the effects of the sigma-1 antagonists

capsaicin-induced responses by sigma-1 receptors led to further research in neuropathic pain, since central sensitization is a key feature of this important pathological state [82]. The most significant findings for the effects of sigma-1 antagonists in preclinical models of neuropathic pain are summarized in Table 9.6, and described below.

In experimental animals and humans, peripheral neuropathic pain can be produced by nerve trauma and by the administration of agents with neurotoxic properties, such as antineoplastics (reviewed in [90]). It was shown that the repeated systemic administration of the selective sigma-1 antagonist S1RA, starting before neuropathy wbvvas established (immediately after the injury, i.e. preventive treatment), prevented the development of sensory hypersensitivity that characterizes neuropathic pain (mechanical and cold allodynia and heat hyperalgesia) [11]. These effects of the sigma-1 antagonist disappeared after treatment was discontinued, and repeated administration did not induce tolerance to the antihypersensitivity to mechanical or thermal stimuli [11]. The ameliorative effects of S1RA on neuropathic hypersensitivity were not limited to its preemptive administration, since systemic administration of this sigma-1 antagonist was able to fully reverse sensory hypersensitivity once the neuropathy was fully established (several days after the injury, i.e. curative treatment) [11, 91, 92]. Importantly, neuropathic rodents were shown to freely self-administer S1RA once neuropathy was established, as a way to reverse not only mechanical and cold allodynia and heat hyperalgesia, but also neuropathic anhedonia (measured as decreased preference for a sweetened liquid) as an indicator of the negative emotional state induced by pain [92]. These results in rodents reflect the efficacy of treatment as evidenced by both the voluntary self-administration of S1RA and the marked positive effects on the animal’s emotional state.

Table 9.6 Summary of the effects on neuropathic pain of sigma-1 receptor inhibition by the systemic administration of sigma-1 antagonists in wild-type animals or in sigma-1 knockout mice. Mechanical allodynia was assessed with von Frey filaments, cold allodynia was evaluated with a cold plate or by the evaporation of an acetone drop on the affected paw, and heat hyperalgesia was evaluated with a hot plate or a Hargreaves device. For purposes of comparison, sigma-1 knockout are classified as "preventive" treatment, since sigma-1 inhibition was present before the peripheral nerve injury

Injury	Readout	Sigma-1 antagonist/KO	Route	Preventive / Curative	Effect on allodynia/hyperalgesia	References
PSNL	Heat hyperalgesia	KO	–	Preventive	No effect	[83]
		S1RA	Systemic	Preventive	Attenuation	[11]
				Curative		[91, 92]
	Cold allodynia	KO	–	Preventive	Attenuation	[83]
		S1RA	Systemic	Preventive	Attenuation	[11]
				Curative		[92]
	Mechanical allodynia	KO	–	Preventive	Attenuation	[83]
		S1RA	Systemic	Preventive	Attenuation	[11]
				Curative		[91, 92]
	Anhedonia	S1RA	Systemic	Curative	Attenuation	[92]
Paclitaxel	Mechanical allodynia	KO	–	Preventive	Attenuation	[97, 98]
		S1RA	Systemic	Preventive	Attenuation	[97]
		BD-1063				[97, 98]
		S1RA	Systemic	Curative	Attenuation	[97]
		BD-1063				
	Cold allodynia	KO	–	Preventive	Attenuation	[97, 98]
		S1RA	Systemic	Preventive	Attenuation	[97]
		BD-1063				[97, 98]
		S1RA	Systemic	Curative	Attenuation	[97]
		BD-1063				
CCI	Mechanical allodynia	BD-1047	i.t.	Preventive	Attenuation	[5, 93–96]
		BD-1047		Curative	No effects	[5, 94, 95]
	Heat hyperalgesia	BD-1047	i.t.	Preventive	No effect	[5, 93–96]
		BD-1047		Curative		[5, 94, 95]

PSNL partial sciatic nerve ligation, *CCI* chronic constriction injury, *i.t.* intrathecal, *KO* knockout

Further experiments were done in sigma-1 KO mice with traumatic nerve injury. The findings were consistent with those obtained with pharmacological treatments: mutant mice did not develop signs of either cold or mechanical allodynia, although they showed heat hyperalgesia [83]. The ability of S1RA to prevent neuropathic heat hyperalgesia and the normal development of this sensory alteration in injured sigma-1-KO mice suggest once again that compensatory mechanisms in specific pain pathways might develop in sigma-1-KO mice.

Further studies were done to examine the role of spinal sigma-1 receptors in peripheral neuropathic pain. It was shown that sigma-1 receptor protein was transiently upregulated in the dorsal spinal cord in the early days after traumatic nerve injury [5, 93], and that the repeated i.t. administration of the sigma-1 antagonist BD-1047 in the early days after nerve injury prevented the full development of neuropathic mechanical allodynia [5, 94]. These results underline the importance of spinal sigma-1 receptors in the development of neuropathic hypersensitivity. In fact, in neuropathic animals sigma-1 inhibition decreased phosphorylation of the NR1 subunit of NMDA receptors in the spinal cord [5], reduced the phosphorylation of extracellular signal-regulated kinase (ERK1/2) [83], and decreased the production of reactive oxygen species [94]. The role of

these phenomena in the process of central sensitization is well known [82]. Interestingly, it was recently reported that sigma-1 receptor antagonism also decreases the activation of spinal cord astrocytes, which are known to participate in neuronal sensitization [93, 95, 96]. This finding suggests that the effects of sigma-1 receptors on pain may not be restricted to neuronal modulation, but may operate in other cell types.

Although the role of spinal sigma-1 receptors in the development of neuropathic mechanical allodynia is clear, the i.t. administration of the sigma-1 antagonist BD-1047 during the induction phase of neuropathy failed to alter thermal hyperalgesia [5, 93–96]. When the sigma-1 antagonist was given i.t. after the neuropathy was fully established, it had no effect on either mechanical or thermal hypersensitivity [5, 94, 95]. These results are in marked contrast to the previously noted efficacy of systemically administered sigma-1 antagonists in both mechanical and thermal hypersensitivity either before the neuropathy was established or when it was fully developed [11, 91, 92, 97, 98]. Together, these contrasting observations suggest that in addition to the participation of sigma-1 receptors at the spinal level, these receptors might also contribute at other locations to the amelioration of the neuropathic pain phenotype produced by systemically administered sigma-1 antagonists. In this connection, it has been shown that sigma-1 antagonists induce the dissociation of sigma-1 receptors and NMDAR NR1 subunits in the PAG of animals with traumatic nerve injury, and this process may have an inhibitory effect on NMDAR activity [17]. Therefore, the modulation of sigma-1 receptors at supraspinal sites may contribute to the wider suite of actions of systemically administered sigma-1 antagonists compared to the i.t. administration of these drugs.

Further studies have investigated the neuropathy induced by paclitaxel, a first-line chemotherapeutic agent used to treat several types of cancer, but which frequently produces painful peripheral neuropathies as one of its major side effects [99]. As in the results found after traumatic nerve injury, systemic treatment with the sigma-1 antagonists BD-1063 and S1RA abolished mechanical and cold allodynia once neuropathy was fully developed [97]. Importantly, sigma-1 KO mice or wild-type mice treated preemptively (during administration of the taxane) with sigma-1 antagonists did not develop paclitaxel-induced neuropathic pain, and the sensory abnormalities of neuropathy did not manifest even after treatment with the sigma-1 antagonists was discontinued [97, 98]. These behavioral changes can be attributed to the decrease in mitochondrial abnormalities (as a sign of the toxicity) in myelinated A-fibers [98], which are thought to play a major role in the neuropathic pain induced by this antineoplastic (e.g. [100]). These results indicate that peripheral sigma-1 receptors play a pivotal role on the neuronal toxicity induced by the taxane.

In summary, sigma-1 receptors clearly modulate central sensitization during neuropathic pain. Although most studies of neuropathic pain and sigma-1 receptors have focused on the role of these receptors at spinal levels, the evidence points to two conclusions: 1) The spinal effects of sigma-1 antagonists do not fully account for the effects seen in animals treated systemically with sigma-1 antagonists, and 2) sigma-1 receptors at other locations (such as in supraspinal structures or in the peripheral nervous system) may contribute to the ameliorative effects of the systemic administration of sigma-1 antagonists on neuropathic pain.

9.3.3 Sigma-1 Receptors and Other Types of Pathological Pain: Inflammatory and Ischemic Pain

Inflammatory pain is a major type of clinical pain (e.g. [101]). In contrast to neuropathic pain, inflammatory pain is characterized by a more pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to the milieu of inflammatory mediators released at the inflammation site [101]. In addition, the pain induced by peripheral ischemia is a distinct type of pain that is also partly mediated by peripheral sensitization mechanisms triggered by prolonged hypoxia [102]. Inflammatory and ischemic pain

are not devoid of central effects, and central sensitization also undoubtedly participates in the generation of these types of pain [82].

In contrast to the many studies focused on sigma-1 receptors and neuropathic pain, the role of these receptors in inflammatory or ischemic pain has only been described very recently, although with promising results. These studies are summarized in Table 9.7 and described below.

Inflammatory pain can be induced in rodents by the administration of proinflammatory agents. The administration of carrageenan or zymosan is used to study acute inflammation, whereas other agents such as complete Freund's adjuvant (CFA) are used to study longer-lasting inflammatory pain hypersensitivity [90, 103]. The systemic administration of several sigma-1 antagonists was reportedly able to prevent acute inflammatory pain hypersensitivity measured as mechanical hyperalgesia [36, 104], mechanical allodynia [105–107] or heat hyperalgesia [36, 104–107] in response to acute inflammatory insult with carrageenan [36, 105–107] or zymosan [104]. In addition, systemically administered S1RA was also shown to reverse the sensory gain (mechanical allodynia) during chronic inflammation induced by CFA, even when this was fully developed [107]. As for other aspects of the pain phenotype described in the preceding sections, sigma-1 KO mice only partially replicated the ameliorative effects induced by sigma-1 antagonists. Inflammatory mechanical hyperalgesia was abolished in sigma-1 KO mice [36], in agreement with the previously noted effects of sigma-1 antagonists. However, KO mice showed thermal hypersensitivity and mechanical allodynia in response to inflammation, to a similar extent to that seen in wild-type animals [36, 107]. This absence of effects by sigma-1 KO, does not match the effects of sigma-1 antagonists (see Table 9.7 for details). However, pharmacological sigma-1 antagonism did not have any effect in sigma-1 KO mice [36, 107]. These findings indicated once again that the presence of sigma-1 receptors is necessary for the ameliorative effects of the drugs.

It was recently shown that the systemic administration of BD-1047 decreased phosphorylation of the NMDAR NR1 subunit, and also decreased microglia density and IL-1β release in the spinal cord of animals with inflammation [104]. In addition, the spinal administration of this sigma-1 antagonist was able to fully reverse the mechanical and thermal hyperalgesia induced by inflammation [104]. These results clearly indicated that spinal sigma-1 receptors may play an important role in the development of inflammatory pain. It was also shown that peripheral pharmacological sigma-1 antagonism (by BD-1063 and S1RA) at the site of inflammation was sufficient to fully abolish inflammatory hyperalgesia [36], indicating that the activity of peripheral sigma-1 receptors is needed to produce the sensory alterations induced by painful inflammation. This line of evidence showed that the sigma-1 antagonist BD-1047, when administered peripherally before hypersensitivity developed (but not once it was fully developed), was able to prevent the sensory gain induced by ischemia [9]. Interestingly, treatment with this sigma-1 antagonist showed a synergistic effect with amiloride, an ASIC blocker [9]. This synergistic effect can be explained by the direct protein–protein interaction between sigma-1 receptors and ASIC (as detailed in Sect. 1.1). In addition, BD-1074 also had a synergistic antiallodynic effect with TNP-ATP, a P2X purinoreceptor antagonist [9]. Although a direct interaction between sigma-1 and P2X receptors is plausible, it has not been demonstrated yet. In addition to ASIC and P2X receptors, other unexplored possible targets are important in peripheral sensitization and may be susceptible to modulation by sigma-1 receptors. For example, sigma-1 receptors are known to modulate the signaling of bradykinin and nitric oxide (reviewed in [108]). Therefore, the effects of sigma-1 antagonists on peripheral sensitization may be more complex, and further studies are needed to elucidate the mechanism of action and therapeutic potential of these drugs.

In summary, these recent studies expand the therapeutic possibilities of sigma-1 receptors beyond neuropathic pain states, by showing that both central and peripheral sigma-1 receptors may be targeted for the relief of inflammatory and ischemic pain.

Table 9.7 Summary of the effects of sigma-1 receptor inhibition by sigma-1 antagonists in wild-type animals, or in sigma-1 knockout mice, on inflammatory and ischemic sensory hypersensitivity. Mechanical hyperalgesia was assessed as sensitivity to paw pressure, mechanical allodynia was evaluated with von Frey filaments, and heat hyperalgesia was assessed with the Hargreaves method. For comparison, sigma-1 knockout mice are classified as "preventive" treatment, since sigma-1 was inhibited before inflammation or ischemia was induced

Pain type	Experimental procedure	Readout	Sigma-1 antagonist/ KO	Route	Preventive/Curative	Effect	References
Inflammation	Carrageenan (i.pl.)	Mechanical hyperalgesia	S1RA	Systemic	Preventive	Attenuation	[36]
				i.pl.			
			BD-1063	Systemic			
				i.pl.			
			KO	-	Preventive	Attenuation	
		Mechanical allodynia	(−)-MRV3	Systemic	Preventive	Attenuation	[105]
			(+)-MR200				[106]
			S1RA				[107]
			KO	-	Preventive	No effect[a]	[107]
		Thermal hyperalgesia	S1RA	Systemic	Preventive	Attenuation	[36, 107]
				i.pl.			[36]
			BD-1063	Systemic	Preventive	Attenuation	[36]
			BD-1063	Systemic	Preventive	Attenuation	[36]
				i.pl.			
			(−)-MRV3	Systemic	Preventive	Attenuation	[105]
			(+)-MR200	Systemic		Attenuation	[106]
			KO	-	Preventive	No effect[a]	[36, 107]
	Zymosan (i.pl.)	Mechanical hyperalgesia	BD-1047	Systemic	Preventive	Attenuation	[104]
				i.t.			
		Thermal hyperalgesia	BD-1047	Systemic	Preventive	Attenuation	[104]
				i.t.			
	CFA (i.pl.)	Mechanical allodynia	S1RA	Systemic	Curative	Attenuation	[108]
			KO	-	Preventive	No effect	[108]
Ischemia	$FeCl_2$ in the femoral artery	Mechanical allodynia	BD-1047	i.pl.	Preventive	Attenuation	[9]
					Curative	No effect	

CFA complete Freund's adjuvant, *i.pl.* intraplantar, *i.t.* intrathecal, *KO* knockout

[a]Although sigma-1 KO animals showed mechanical allodynia and thermal hyperalgesia to a degree equivalent to that seen in wild-type mice, sigma-1 antagonists induced a clear antihyperalgesic effect in wild-type mice but not in mutant animals, indicating that on-target mechanisms were involved

9.3.4 Are Sigma-1 Agonists Pronociceptive?

Although the antinociceptive potential of sigma-1 antagonists is unquestioned, the pronociceptive effects of sigma-1 agonists are controversial. Selective sigma-1 agonists (such as PRE-084 or (+)-pentazocine) did not alter sensory thresholds when administered systemically [36–38, 46], supraspinally (e.g. [57]) or peripherally, and at doses able to reverse the effect of sigma-1 antagonists (i.e. able to interact with sigma-1 receptors). Therefore, sigma-1 agonism may be insufficient to sensitize the system to pain. However, the i.t. administration of sigma-1 agonists triggered mechanical allodynia and thermal hyperalgesia [94, 95, 109–112] by acting through inverse mechanisms to those ameliorated by sigma-1 inhibition in neuropathic animals [94, 95, 109–112]. In addition, it was recently reported that the local administration of PRE-084, although devoid of effect when administered alone, synergistically enhanced the pronociceptive effects of low pH (which activates ASIC) and α-β-methylene-ATP (an agonist of P2-purinoreceptors) [9]. These results may indicate that sigma-1 activation alone is not sufficient to bring about pain sensitization, and that priming the nociceptive system may allow sigma-1 receptors to promote sensory hypersensitivity. One explanation for the proalgesic effects of spinal sigma-1 agonism is that i.t. administration might prime the nociceptive system, thus facilitating the pronociceptive effect of sigma-1 agonism. Whether sigma-1 agonists are pronociceptive or not is a moot question, as several drugs are currently marketed that are in fact sigma-1 agonists, e.g. the antitussive carbetapentane or the antidepressant fluvoxamine [3]. Accordingly, an area that warrants further investigation is whether these drugs facilitate sensory hypersensitivity after priming of the nociceptive system.

9.4 Conclusions and Final Remarks

At present, sigma-1 receptors are a promising pharmacological target for pain treatment. Most evidence to date has dealt with the therapeutic possibilities of sigma-1 antagonists for the treatment of neuropathic pain, and as opioid adjuvants that might make it possible to lower the dose of opioids needed to achieve therapeutic effects, and thus minimize their side effects. However, there is recent evidence for other possible indications in the treatment of somatic or visceral pain of diverse etiologies, such as inflammatory or ischemic pain. Sigma-1 receptors act by modulating a variety of channels and receptors important in neurotransmission. NMDARs are probably the most prominent target proteins of sigma-1 receptors in their modulation of pain sensitization and the effects of opioids, but additional client proteins for these receptors may also participate in the pain relief brought about by sigma-1 antagonists. Elucidating the full interactome of sigma-1 receptors will greatly increase our understanding of the physiological and pathophysiological functions of these receptors. Although there is still much to learn about the mechanisms of this ligand-operated intracellular chaperone, it is now clear that sigma-1 antagonists can modulate pain neurotransmission at several steps in the pain circuitry, at both the central and peripheral levels.

A particular line of basic research with clinical potential is the use of sigma-1 KO mice to identify the biological functions of sigma-1 receptors. Although in some experimental situations, sigma-1 KO animals produced results similar to those obtained with sigma-1 antagonists in wild-type animals, this has not always been the case. These disparities have been attributed to compensatory mechanisms in the mutant animals, but it is unclear why these mechanisms are triggered only for some, but not all, aspects of the pain phenotype. These phenomena may be due to differences in the role(s) of sigma-1 receptors in the mechanisms underlying each pain state (acute, neuropathic or inflammatory) and in the pathways involved in each sensory modality (mechanical or thermal). Such differences suggest that the influence of sigma-1 receptors differs – and may involve different protein partners – depending on the pain pathway and its sensitization state. Regardless of the precise nature of the purported compensatory mechanisms, KO mice have been successfully used to test the specificity of drug effects by exploring the anticipated

lack of activity of sigma-1 drugs in the absence of sigma-1 receptors.

In summary, we have reviewed the preclinical evidence from studies designed to test the role of sigma-1 receptors in pain. In light of our current knowledge, it seems clear that there is a persuasive body of evidence in support of the analgesic potential of sigma-1 antagonists. These drugs have a distinct mechanism of action and represent a new avenue of research in the treatment of pain from different causes. The results of ongoing clinical trials with selective sigma-1 receptor antagonists to treat painful conditions are therefore eagerly awaited.

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Sigma-1 Receptors and Neurodegenerative Diseases: Towards a Hypothesis of Sigma-1 Receptors as Amplifiers of Neurodegeneration and Neuroprotection

10

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Abstract

Sigma-1 receptors are molecular chaperones that may act as pathological mediators and targets for novel therapeutic applications in neurodegenerative diseases. Accumulating evidence indicates that sigma-1 ligands can either directly or indirectly modulate multiple neurodegenerative processes, including excitotoxicity, calcium dysregulation, mitochondrial and endoplasmic reticulum dysfunction, inflammation, and astrogliosis. In addition, sigma-1 ligands may act as disease-modifying agents in the treatment for central nervous system (CNS) diseases by promoting the activity of neurotrophic factors and neural plasticity. Here, we summarize their neuroprotective and neurorestorative effects in different animal models of

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acute brain injury and chronic neurodegenerative diseases, and highlight their potential role in mitigating disease. Notably, current data suggest that sigma-1 receptor dysfunction worsens disease progression, whereas enhancement amplifies pre-existing functional mechanisms of neuroprotection and/or restoration to slow disease progression. Collectively, the data support a model of the sigma-1 receptor as an amplifier of intracellular signaling, and suggest future clinical applications of sigma-1 ligands as part of multi-therapy approaches to treat neurodegenerative diseases.

Keywords
Neurorestoration • Stroke • Parkinson's disease • Alzheimer's disease • Amyotrophic lateral sclerosis

10.1 Introduction

Neurodegeneration is characterized by the loss of neuronal integrity, in both structure and function, and can result from acute injury or chronic disease progression. Neurodegenerative diseases are a major cause of morbidity and mortality among the aging population worldwide. The World Alzheimer Report, for example, estimates 46.8 million people worldwide are living with dementia as of 2015 [1]. Based on current trends, this number is projected to almost double about every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050 [1, 2], making efforts to understand and treat these conditions crucial to maintaining the health of an increasingly large demographic. While current treatments for neurodegenerative conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and stroke can temporarily alleviate some symptoms and improve quality of life, they are generally ineffective at slowing or stopping disease progression.

Sigma receptors are increasingly recognized targets for novel therapeutic intervention in neurodegenerative conditions [3]. These proteins are implicated in diverse neural mechanisms, including the modulation of cell survival and function, calcium signaling, neurotransmitter release, inflammation, and synaptogenesis [4–6]. The two established subtypes of sigma receptors, sigma-1 and sigma-2, are highly expressed in the central nervous system (CNS), and are distinguishable pharmacologically, functionally, and by molecular size [7].

The present chapter focuses on putative neuroprotective roles conferred by sigma-1 receptor activity as this receptor is the better characterized of the two subtypes. If both subtypes may be involved in an effect described here, we refer to sigma receptors in general, rather than specifying the subtype. We begin with a brief overview of the neuroprotective and restorative effects of sigma-1 ligands in various animal models of neurodegenerative diseases. "Neuroprotection" and "neurorestoration" are terms that can be interpreted in a variety of ways. In the context of this review, neuroprotection is any sequence of events that interrupts or slows the sequence of injurious biochemical and molecular events that, if left unchecked, would likely lead to cell damage and/or loss. Neurorestoration is the regeneration of functional tissue, which is impacted by the capacity of surviving cells to adapt after injury and of new cells (through neurogenesis and/or recruitment of glial cells to damaged areas) to support repair. This is followed by a summary of how sigma-1 ligands may confer their therapeutic effects by modulating mechanisms that are common across a wide array of neurodegenerative conditions, including excitotoxicity, Ca^{+2} dysregulation, mitochondrial and endoplasmic reticulum (ER) dysfunction, neuroinflammation, and

reactive gliosis. In addition, sigma-1 ligands may promote neurorestorative processes to enhance the structure and function of neurons that become compromised in disease, or to stimulate the influx of new cells to assist in repairing damage to the nervous system. Finally, we discuss the model of the sigma-1 receptor as an "amplifier" of intracellular signaling, the resulting ways that sigma-1 receptors may be involved in disease and therefore exploited therapeutically, and the potential application of sigma-1 ligands as part of combined therapeutic approaches in future clinical studies of neurodegenerative diseases.

10.2 Sigma-1 Receptor Ligands in Animal Models of Neurodegenerative Diseases

In general, in model systems of neurodegenerative diseases, deficits in sigma-1 receptor level or activity are associated with neurodegeneration, while sigma-1 receptor activation or overexpression are associated with neuroprotection. Consistent with this, most reports of beneficial effect come from studies of sigma-1 agonists, with these effects generally showing sensitivity to sigma-1 antagonism. Below, we highlight the neuroprotective effects of sigma-1 receptors in several animal models of neurodegenerative disease. In our recent review, we highlight additional models where targeting sigma-1 receptors alters neurodegenerative disease processes [3].

10.2.1 Stroke

Acute brain injury following cerebral ischemia (stroke) and trauma can lead to long-term neurological and psychiatric deficits. As the primary insult (e.g., direct mechanical damage) cannot be therapeutically influenced, the goal of treatment is to limit secondary injury processes. Following cerebral ischemia, both necrotic and apoptotic cell death can be induced through complex interactions of pathological processes, including excitotoxicity and inflammation [8, 9]. Neuroprotective and neurorestorative effects of sigma-1 agonists (e.g., decreasing cell death, protecting against tissue damage, and increasing synaptic protein expression) have been shown in multiple animal models of stroke, including mouse [10], rat [11–16], gerbil [17] and cat [18]. In rat models of stroke, for example, decreased infarct volume as well as enhanced neuronal survival were observed following acute treatment with a sigma agonist 24 h after the onset of ischemia [14, 15]. In addition, functional recovery with or without changes in infarct volume was observed when sigma agonists were administered as late as 2 days post-stroke [15, 19]. The potential to treat at extended times following the initial embolic injury warrants further investigation, as the only available post-stroke treatment approved for use in humans is thrombolytics, which is limited to 4 h post-stroke due to the risk of hemorrhagic transformation (i.e., conversion of an ischemic stroke to a hemorrhagic one following reperfusion) [20]. Of note, Ruscher and colleagues demonstrated that treatment of rats subjected to permanent or transient middle cerebral artery occlusion (MCAO) with the selective sigma-1 agonist SA4503 (1-[2-(3,4-dimethoxyphenyl) ethyl]-4-(3-phenylpropyl) piperazine) starting 2 days after injury conferred significantly better recovery rates of sensorimotor function compared with the vehicle group [19]. The significant improvement of neurological function following MCAO was associated with increased levels of the synaptic proteins neurabin and neurexin in the peri-infarct area [19]. This improvement was sustained 2 weeks after discontinuation of SA4503 [19]. These results suggest that stimulation of sigma-1 receptors promote neural adaptations (e.g., increases in synaptic proteins and potentially synaptic connections) to facilitate recovery following MCAO [19].

10.2.2 Other Acute CNS Injury

Additional beneficial effects of sigma agonists have been reported in other models of acute

CNS injury. Following spinal root avulsion in adult rats, administration of the sigma-1 agonist PRE084 (2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) promoted motor neuron survival [21]. Co-administration of the sigma-1 antagonist BD1063 (1-[2-(3,4-dichlorophenyl) ethyl]-4-methylpiperazine) blocked this effect [21]. In another study, the sigma agonist PPBP (4-phenyl-1-(4-phenylbutyl) piperidine) improved neurological function and reduced striatal cell death when administered after global hypoxia-ischemia (induced by asphyxic cardiac arrest followed by resuscitation) in newborn piglets [22]. An additional study showed that the sigma-1 agonist PRE084 reduced cortical lesion size and cell death following excitotoxic perinatal brain injury in newborn mice [23]. However, confirmation of a sigma-1-mediated mechanism using an antagonist was not tested in the two aforementioned studies involving neonates.

10.2.3 Amyotrophic Lateral Sclerosis

In a superoxide dismutase 1 (SOD1)-G93A mouse model of amyotrophic lateral sclerosis (ALS), daily administration of the selective sigma-1 agonist PRE084 from 8 to 16 weeks of age improved spinal motor neuron function and survival, demonstrated by the preservation of neuromuscular connections and motor neuron number in the spinal cord, maintenance of muscle action potential amplitudes, and improvement in locomotor performance [24]. This attenuation of the disease state was associated with an increase in survival time in PRE084-treated mice compared to controls [24]. In contrast, genetic ablation of sigma-1 receptors accelerated the appearance of motor deficits as well as decreased longevity in the SOD1-G93A mouse model [25].

Sigma-1 agonists may also be effective in cases of SOD-1-independent mechanisms of ALS. Chronic treatment with PRE084 improved motor neuron survival and locomotor performance in the wobbler mouse, which is a model of motor neuron degeneration [26].

10.2.4 Parkinson's Disease

In an intrastriatal 6-hydroxydopamine (6-OHDA) lesion model of Parkinson's disease (PD), mice were treated daily with PRE084 for 5 weeks, starting on the same day as the lesion induction [27]. PRE084 gradually and significantly improved spontaneous forelimb use, along with a partial recovery of dopamine levels and increased dopaminergic fiber densities, compared to saline-treated animals [27]. PRE084 treatment also upregulated neurotrophic factor protein levels and increased activation of their downstream effector pathways [27], further suggesting that sigma-1 receptor activation contributes to the restoration of synaptic connectivity and functional recovery in neurodegeneration disease models.

10.2.5 Alzheimer's Disease

In an amyloid beta (25–35) peptide-induced mouse model of Alzheimer's disease (AD), selective and non-selective sigma-1 agonists improved both molecular and behavioral markers of neurodegeneration [2, 28, 29]. The selective sigma-1 agonist PRE084 and the non-selective sigma-1 agonists donepezil or AVANEX2–73 mitigated spatial working memory deficits in spontaneous alternation tests [2, 28]. They also attenuated contextual long-term memory in the step-through passive avoidance procedure [2, 28]. These effects were mediated, at least in part, by sigma-1 receptors, demonstrated by their sensitivity to the sigma-1 antagonist BD1047 (N-[2-(3, 4-dichlorophenyl) ethyl]-N-methyl-2-(dimethylamino) ethylamine) [2, 28]. In addition, treatment with these sigma-1 agonists decreased amyloid beta-induced lipid peroxidation in the hippocampus, consistent with a role in decreasing oxidative damage; these protective effects were also attenuated by BD1047 [2, 28]. In amyloid beta (25–35)-treated mice exhibiting cognitive deficits, PRE084 or igmesine, another selective sigma-1 agonist, showed greater antidepressant efficacy compared to non-amyloid beta-treated animals [6]. This enhanced efficacy was

not seen with the classic antidepressants desipramine or fluoxetine, suggesting that selective sigma-1 receptor agonists are promising alternatives for alleviating the depressive symptoms in AD patients.

10.2.6 Possible Therapeutic Effects of Sigma-1 Antagonism

There are a few studies that suggest a potential benefit of sigma-1 antagonism to promote neuroprotection, though the specific effects of sigma-1 suppression are unclear. The putative sigma-1 antagonist haloperidol, for instance, reduced infarct volume in a rat model of MCAO [30]. When compared to eight other butyrophenone compounds in an *in vitro* assay of glutamate-induced oxidative stress, the authors found a significant positive correlation between haloperidol's protective potency (i.e., nanomolar vs. micromolar concentration required to increase cell survival) and affinity for sigma-1 receptors [30]; however, haloperidol also has similar nanomolar affinity to other targets, including dopamine, serotonin (5-HT), and alpha adrenergic receptors, making it difficult to attribute its primary effect to sigma-1 antagonism in these models. More selective sigma antagonists have been shown to reduce methamphetamine (METH)-induced neurotoxicity [31] and alleviate neuropathic pain [32]. In wildtype mice, knockout of sigma-1 receptors prevented subchronic administration of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) from causing the motor and histochemical deficits characteristic of PD [33]. This protective effect, however, was not observed in sigma-1 knockout mice [33], suggesting the importance of these receptors in the etiology of the disease. While these and other studies leave open the possibility that sigma-1 antagonism may be beneficial in certain conditions, there is much stronger and more direct evidence that sigma-1 activation is neuroprotective, and therefore that sigma-1 agonist-based therapeutics are more likely to protect against neurodegeneration than antagonists.

10.3 Sigma-1 Receptor Mediated Mechanisms of Neuroprotection

While neurodegenerative diseases are a heterogeneous group of illnesses with distinct clinical phenotypes and diverse etiologies, emerging evidence suggests that they share important pathogenic mechanisms, including excitotoxicity [20, 34, 35], Ca^{2+} dysregulation [36, 37], mitochondrial and ER dysfunction [38–41], inflammation [42, 43], and in some cases, astrogliosis [44]. In addition, neurotrophic factors and neural plasticity have been found to be important targets for disease-modifying treatments for CNS diseases [45–48]. In this section, we focus on the ways in which sigma-1 receptor activity may modulate these mechanisms to elicit neuroprotection.

10.3.1 Glutamate Excitotoxicity

Excitotoxicity occurs when high levels of glutamate cause persistent activation of N-methyl-D-aspartate (NMDA) receptors, allowing an influx of Ca^{2+} that can activate downstream mechanisms of programmed cell death, including the activation of calpains, proteases, protein kinases, nitric oxide synthase (NOS) and the mitochondrial permeability transition pore [34, 49]. Excitotoxicity has been observed in multiple neurodegenerative disease states, including ALS, AD, PD, stroke and METH toxicity [20, 26, 35, 50, 51]. Through the modulation of glutamate and its receptors, sigma ligands have been reported to be neuroprotective against excitotoxicity in retinal ganglion cells (RGCs), primary neuronal cultures, and ischemic stroke models [23, 52–57].

The mechanisms by which sigma ligands modulate excitotoxic glutamate release are poorly understood. However, studies to date implicate multiple mechanisms. In a chronic restraint stress model of depression, for example, stimulation of sigma-1 receptors enhanced glutamate release by increasing presynaptic cytoplasmic release of Ca^{2+} from ER stores [58]. Sigma-1 agonists also inhibited the release of glutamate

evoked by a K^+ channel blocker in cortical nerve endings, in a sigma-1 antagonist-sensitive manner [59]. In addition, treatment with sigma-1 agonists has led to decreased Ca^{2+} entry through presynaptic voltage-dependent Ca^{2+} channels and the suppression of protein kinase C (PKC) signaling cascades, resulting in decreased glutamate release from nerve terminals in the rat cerebral cortex [59].

In addition to influencing glutamate release, sigma-1 receptor activity is implicated in the neuronal responses to NMDA receptor stimulation, both directly, through interactions with specific subunits of the NMDA receptor [60, 61] and indirectly, through the modulation of other ion channels [62]. Sigma-1 receptors have been shown to bind to the cytosolic C-terminal region of the NMDA receptor NR1 subunit in recombinant cells, which can be inhibited by sigma-1 antagonists [63]. Activation of sigma-1 receptors can also increase the interaction between sigma-1 receptors and NR2 subunits of NMDA receptors. This happens concurrently with increased translocation to the cell surface and results in an increase in NMDA receptor availability at the plasma membrane [64]. The authors hypothesized that the relationship between sigma-1 receptors and NR2 subunits is therefore an indirect one, involving direct interactions between sigma-1 receptors and NR1 subunits that are part of the same tetrameric NMDA receptor complex as the NR2 subunits being probed [64]. In another study, activation of sigma-1 receptors induced phosphorylation of NR1 subunits and subsequent potentiation of NMDA receptor function in spinal neurons by modulating PKC signaling via the alpha and epsilon isoforms of PKC [65].

Sigma-1 receptor activation can also affect the interaction of other proteins with NMDA receptors to elicit neuroprotective effects. For example, sigma-1 agonists enhanced the interaction of histidine triad nucleotide binding protein 1 (HINT1) with G-protein coupled receptors (GPCRs) and in turn stimulated GPCR-NMDA interactions, promoting protective effects against excitotoxicity [66]. Downstream of influencing NMDA receptor function and/or activity, sigma-1 agonists have been shown to be neuroprotective by increasing brain-derived neurotrophic factor (BDNF) levels in an ischemia/reperfusion vascular dementia model [67]. This appeared to be mediated through NR2A-CAMKIV (calcium/calmodulin-dependent protein kinase type IV)-TORC1 (transducer of regulated cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB) activity) pathways [67].

In addition to NMDA receptors, sigma receptors may regulate (directly or indirectly) other glutamatergic targets including kainate and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) glutamate receptors to confer neuroprotective effects. For example, sigma-1 agonists attenuated kainate receptor-induced hippocampal neurotoxicity and seizures by acting downstream and decreasing c-fos/c-jun expression and activator protein (AP)-1 DNA-binding activity [68, 69]. Sigma-1 agonism also afforded neuroprotection by reducing the expression of AMPA receptors in cultured cortical neurons, possibly via decreasing activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling [70], a critical pathway for the maintenance of ionotropic glutamate receptors [71].

10.3.2 Ca^{2+} Dysregulation

High and persistent Ca^{2+} release may contribute to neurotoxicity and cell death. In addition to Ca^{2+} flux through NMDA receptors, there are several additional means by which Ca^{2+} levels can be increased to toxic levels in neurons, including exit from intracellular ER and mitochondria stores, influx through voltage-dependent plasma membrane Ca^{2+} channels, Na^{2+}/ Ca^{2+} exchangers, and acid-sensing ion channels (ASICs) [49].

Sigma-1 agonists have been shown to regulate intracellular Ca^{2+} levels and prevent the increased expression of pro-apoptotic genes and caspases in RGCs [72], as well as in rat cortical neurons with prolonged exposure to amyloid beta peptide [73]. These molecular effects correspond with phenotypic improvements to memory impairments in animal models [29].

In both physiological and pathophysiological conditions, sigma-1 receptors appear to function as chaperones and Ca^{2+} sensors [5, 74–77]. At the ER mitochondrial-associated membrane (MAM), sigma-1 receptors play an important role in regulating Ca^{2+} levels via inositol trisphosphate (IP_3) receptors and maintaining intracellular Ca^{2+} homeostasis [76].

In addition to modulation of intracellular sources of Ca^{2+}, sigma-1 receptors can alter the behavior of plasma membrane ion channels, thereby altering Ca^{2+} uptake into the cell. Sigma-1 agonists have been shown to mediate the elevated intracellular Ca^{2+} levels caused by activation of ASIC-1a during stroke-induced ischemia [78]. Among the Ca^{2+}-associated downstream signaling pathways, sigma-1 agonism reduced the activation of the MAPK/ERK pathway, affording neuroprotection [70]. In rat primary ganglion cells, the sigma-1 agonist (+)-SKF10047 ((2S,6S,11S)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol) inhibited potassium chloride (KCl)-induced Ca^{2+} influx through L-type Ca^{2+} channels, which was reversed by the sigma-1 antagonist, BD1047. This inhibition involved direct interactions between L-type Ca^{2+} channels and sigma-1 receptors [79]. In addition to their effects on neurons, sigma-1 ligands can suppress microglial activation through Ca^{2+}-dependent mechanisms, decreasing the release of inflammatory cytokines [80].

10.3.3 ER Stress

Neurodegenerative conditions associated with ER stress include METH toxicity, HD, AD, ALS and PD [38, 40, 81]. One result of ER dysfunction is the accumulation of unfolded or misfolded proteins within the ER lumen. This accumulation activates the unfolded protein response (UPR), which occurs through three major signaling pathways: protein kinase RNA like ER kinase (PERK), inositol requiring enzyme 1 alpha (IRE1α), and activating transcription factor 6 (ATF6). As chaperones, sigma-1 receptors participate closely in the degradation of unfolded proteins [82], and multiple studies describe sigma-1 receptor modulation of the UPR [76, 83, 84]. Moreover, the C-terminus on the sigma-1 receptor has been shown to interact with the glucose-regulated protein 78 (GRP78)/immunoglobulin heavy-chain binding protein (BiP) [85], a critical regulator of all three arms of the UPR [86]. Following the administration of the Ca^{2+} channel inhibitor thapsigargin or the GPT (UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosamine-1-phosphate transferase) inhibitor tunicamycin, which are frequently used to model ER stress and induce UPR in cell culture models, sigma-1 receptor expression is upregulated in response to activation of the PERK pathway [87] and more specifically, ATF4, a downstream target of PERK signaling [88]. This upregulation of sigma-1 receptor expression found in HEK293 (human embryonic kidney) and Neuro2a (mouse neuroblastoma) cells can repress cell death signals that accompany ER stress [87]. Consistent with this, overexpression of the sigma-1 receptor decreased the activation of PERK and ATF6 and increased cell survival in Chinese hamster ovary (CHO) cells, whereas knockdown of sigma-1 receptors destabilized the conformation of IRE1 and decreased cell survival following administration of thapsigargin [76, 84].

Not surprisingly, treatment with the selective sigma-1 agonist SA4503 mitigated ER stress and reduced cell death in the retina following light-induced damage [89]. Using the selective 5-HT reuptake inhibitor (SSRI) fluvoxamine, a potent sigma-1 agonist that exhibits stronger affinity for sigma-1 receptors than other SSRIs [90], Omi and colleagues also showed that fluvoxamine, via activation of sigma-1 receptors, upregulated sigma-1 receptor expression and inhibited cell death in Neuro2a cells exposed to tunicamycin (which disrupts protein folding and directly induces the UPR) [88]. The specificity of sigma-1 involvement was confirmed with the addition of the sigma-1 antagonist NE100 (N, N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy) phenyl] ethylamine monohydrochloride), which blocked the effects of fluvoxamine [88].

10.3.4 Mitochondrial Ca^{2+} Uptake and Activity

In their quiescent state, sigma-1 receptors are located in the mitochondrial-associated membrane (MAM) in association with the ER chaperone protein BiP, and under these conditions are inactive. Activation by ligand binding or various pathways of ER stress causes the dissociation of sigma-1 receptors from BiP and allow their participation in multiple downstream pathways.

Within the MAM, activated sigma-1 receptors appear to stabilize IP_3 receptors by protecting them from proteasomal degradation [76] and activate them by promoting their dissociation from the ion channel chaperone protein ankyrin B 220 [91]. This promotes Ca^{2+}-induced Ca^{2+} release from the ER through Ca^{2+}-activated IP_3 production, and then Ca^{2+} trafficking into the mitochondria. Ca^{2+} uptake into the mitochondrial matrix is a sensitive regulator of oxidative phosphorylation. Sub-micromolar increases in matrix Ca^{2+} directly activate multiple enzymes, including glycerol phosphate dehydrogenase, isocitrate dehydrogenase, and oxoglutarate dehydrogenase, and, indirectly (via dephosphorylation) pyruvate dehydrogenase, resulting in increased flux through the Krebs cycle and facilitating increased rates of oxidative phosphorylation. In this way, Ca^{2+} acts as an interorganellar signal to "tune" ATP supply according to the ATP demand dictated by the rest of the cell.

The sigma-1 receptor-IP_3 receptor interaction may promote mitochondrial Ca^{2+} uptake and, ultimately, cell survival [76]. Consistent with this, Shioda and colleagues identified a truncated splice variant of the sigma-1 receptor (short form sigma-1 or sigma-1S) in the mouse hippocampus that localizes to the MAM and complexes with non-truncated sigma-1 receptors, but does not complex with IP_3 receptors [92]. In Neuro2a C3100 cells, exogenous overexpression of non-truncated sigma-1 receptors enhanced ATP- or IP_3-induced mitochondrial Ca^{2+} uptake whereas overexpression of sigma-1S decreased mitochondrial Ca^{2+} uptake compared to control cells [92]. Following tunicamycin-induced ER stress, the exogenous overexpression of non-truncated sigma-1 receptors protected IP_3 receptor proteins from degradation and enhanced ATP production, promoting cell survival [92]. In contrast, overexpression of sigma-1S enhanced IP_3 receptor degradation and decreased mitochondrial Ca^{2+} uptake, resulting in increased apoptosis [92]. These findings suggest that sigma-1S destabilizes IP_3 receptors and diminishes IP_3 receptor-driven mitochondrial Ca^{2+} uptake through the loss of sigma-1-IP_3 receptor interactions, resulting in impaired ATP production and increased apoptosis [92]. Of note, mutations of sigma-1 receptors have been found in neurodegenerative conditions such as ALS [93, 94]. It will therefore be important to further evaluate how truncated sigma-1 receptors may interfere with normal receptor function to affect mitochondrial stability.

There is also experimental support for sigma-1 receptor-mediated maintenance of bioenergetic homeostasis. Though the effects are likely indirect, sigma-1 receptor activation is reported to preserve bioenergetic function in multiple models, supporting a neuroprotective role. The sigma agonist PPBP appears to stabilize mitochondrial membrane potential in neurons undergoing excitotoxic stress through glutamate exposure. This stabilization was associated with decreased neuronal death [95]. Another agonist, BHDP (*N*-benzyl-*N*-(2-hydroxy-3,4-dimethoxybenzyl)-piperazine), appeared to have "mitochondrial protective" effects in a liver model of ischemia/reperfusion [96].

10.3.5 Neuroinflammation

The primary mediators of neuroinflammation in the CNS are microglia, which are macrophage-derived cells residing in the CNS. Although multiple microglial phenotypes are believed to result from CNS insult, they are typically classified as M1 and/or M2 responses, similar to peripheral macrophages [42]. M1 microglia are traditionally considered pro-inflammatory and tend to be associated with damage to the CNS, while M2 microglia are anti-inflammatory and associated with neuronal repair and regrowth [42, 97]. Sigma-1 receptors, expressed in microglial and

neurons, may modulate microglial activation and dampen neuroinflammation. Indeed, many studies have shown that sigma agonists may affect M1 and/or M2 responses, with most studies to date focusing on the M1 response. For example, Robson and colleagues demonstrated that neurotoxic dosing with METH preferably activated M1 microglia responses within the mouse striatum as represented by significant increases in the pan-macrophage markers, cluster of differentiation 68 (CD68) and ionized calcium binding adapter molecule 1 (IBA-1), without concurrent increases in an M2 marker, CD163 [98]. Pretreatment with the sigma ligand SN79 (6-acetyl-3-(4-(4-(4-fluorophenyl) piperazin-1-yl) butyl) benzo[d] oxazol-2(3H)-one) attenuated the increase in CD68 and IBA-1, indicating prevention of METH-induced M1 microglial activation [98]. Associated with this reduction in M1 microglia was an obviation of IL-6 and oncostatin M, showing protection against neuroinflammation [98]. In lipopolysaccharide (LPS)-stimulated murine microglial BV2 cells, the sigma-1 agonist SKF83959 (6-chloro-2,3,4,5-tetrahydro-3-methyl-1-(3-methylphenyl)-1H-3-benzazepine-7,8-diol) prevented M1 microglial activation and decreased pro-inflammatory cytokines, including tumor necrosis factor alpha, IL-1 beta, and inducible NOS [99]. The sigma agonists DTG (1,3-di-(2-tolyl)guanidine) and afobazole have also been shown to suppress microglial activation and migration and the release of inflammatory cytokines in response to not only LPS, but also other microglial activators such as ATP, uridine triphosphate and monocyte chemoattractant protein-1 [100]. In an *in vivo* model of traumatic brain injury, the sigma-1 agonist PRE084 has been shown to reduce IBA-1 expression following controlled cortical impact in association with reduced lesion volume and improved behavior in mice [101]. Similarly, PRE084 also reduced counts of IBA-1 positive microglial cells in a mouse model of ALS [24].

In contrast, in another study in animals with motor neuron disease, treatment with PRE084 increased the number of cells positive for the pan-macrophage marker CD68 and CD206, which is associated with M2 microglial responses [26]. Sigma ligands also improved microglial cell survival during and at least 24 h after ischemia [100], as well as after toxic exposure to amyloid beta in primary microglia cultures [102]. These data suggest that sigma receptors may modify microglial reactivity to strengthen the reparative microglia phenotype (M2) while attenuating the inflammatory response (M1). Further studies should examine both microglial types to better understand the role of sigma-1 receptors on microglia in neurodegeneration and neurorestoration.

Upon disruption of the blood brain barrier by injury, diapedesis of peripheral leukocytes into the brain may also exacerbate neuroinflammation. Sigma-1 receptors are expressed in lymphocytes, and previous studies have shown the ability of sigma-1 ligands to inhibit CD3 lymphocyte proliferation *in vitro* and LPS-induced release of cytokines *in vivo* [103, 104]. Recently, a novel synthetic, high-affinity and selective sigma-1 ligand was examined in a mouse autoimmune encephalitis model [105], which exhibited histopathological changes characterized by peripheral leukocyte infiltration into the brain along with demyelination and axonal loss [106]. The sigma-1 ligand prevented mononuclear cell accumulation and demyelination in the brain and spinal cord while also increasing the proportion of B-cell subsets and regulatory T-cells, resulting in an overall reduction of the clinical signs of experimental autoimmune encephalitis [105]. Sigma-1 receptors may therefore regulate peripheral immune cells to slow the progression of certain CNS diseases.

10.3.6 Reactive Astrogliosis

Reactive astrogliosis is characterized by the "activation" of astrocytes within the CNS, which may result in the proliferation and migration of astrocytes to areas of damage and in some cases, the formation of glial scars. Glial scar formation is hypothesized to protect surrounding neuronal tissue from further damage as a result of excess inflammation. However, the formation of glial scars also can impede repair and thereby inhibit

the ability of neuronal tracts to regenerate [107]. Sigma receptors are found in astrocytes [7], and accumulating data suggests that sigma-1 receptor activity promotes repair following damage to the CNS. For example, following experimental stroke, a 30 % increase of sigma-1 receptor expression was found in astrocytes, and treatment with a sigma-1 agonist enhanced the recovery of sensorimotor function without decreasing infarct size [19]. This led the authors to suggest that the recovery-promoting action of sigma-1 receptors involved astrocytes in the peri-infarct area [19].

In an animal model of PD, sigma-1 receptor distribution was also noted in astrocytes, in addition to neurons. In the striatum, sigma-1 immunoreactivity predominated in astrocytes (vs. neurons), while in the substantia nigra, sigma-1 immunoreactivity was predominant in dopaminergic neurons (vs. astrocytes) [27]. Following 5 weeks of treatment with the sigma-1 agonist PRE084, there was a shift in distribution of sigma-1 receptors from the neuronal and astrocytic cell bodies into the processes [27]. This occurred in association with functional motor recovery. Consequently, sigma-1 agonists may be increasing astrocytic neuroprotective activity by promoting the intracellular trafficking of sigma-1 receptor proteins and potential transport of other protein partners involved in neuroprotective mechanisms to distal regions of astrocytes.

As well as increasing astrocytic activity, sigma ligands have also been shown to reduce astrocytic activity in some studies. Ajmo and colleagues showed that the sigma agonist DTG reduced astrocyte activation 24 h post-stroke [14]. Penas and colleagues also found that the selective sigma-1 agonist PRE084 reduced astrogliosis and ER stress following spinal root avulsion [108]. PRE084 similarly provided neuroprotection and reduced astrogliosis in preclinical ALS [26] and PD models [27]. Furthermore, in an animal model of METH-induced neurotoxicity, METH increased astrogliosis in the damaged striatum, an effect that could be mitigated with a novel sigma ligand; these effects involved modulation of the OSMR-STAT3 signaling pathway [109], which has been implicated in other neurotoxic conditions such as ischemic stroke or peripheral LPS injections [110].

The ability of sigma ligands to modulate the function of astrocytes, in addition to neurons and microglia, suggest that these ligands may be able to facilitate a coordinated response across cell types to achieve therapeutic outcomes. Additional studies to delineate the interplay between these cell types in the nervous system in health and disease are warranted.

10.4 Sigma-1 Receptor-Mediated Mechanisms of Neurorestoration

In addition to targeting sigma-1 receptors to mitigate neurodegenerative processes, accumulating evidence reveals the potential for sigma-1 agonists to stimulate neurorestorative processes after the CNS has been damaged. This can be achieved either by improving the structural and/or functional integrity of existing cells that have become compromised by disease, or by stimulating the incorporation of new cells into the damaged region to support repair. For example, in a mouse 6-OHDA lesion model of PD, treatment with the sigma-1 agonist PRE084 for 5 weeks after lesion induction can rescue animals from both histological and functional deficits that are normally associated with the lesions [27]. The involvement of sigma-1 receptors in the neurorestorative effects has been confirmed by the inability of PRE084 to evoke similar rescue in sigma-1 receptor knockout mice [27]. Specific neurorestorative mechanisms that can be targeted by sigma ligands are only just beginning to be investigated, and those that have been identified are summarized below.

10.4.1 Increased Growth Factor Expression or Activity

Neurotrophins and growth factors play an integral role in nervous system development, maintenance, and plasticity [111]. Aberrant levels of multiple neurotrophins and growth factors have

been implicated in CNS disorders, including neurodegenerative conditions [112]. These proteins have also been proposed as targets for future therapies [113].

Glia-derived neurotrophic factor (GDNF) has long been known to be capable of rescuing neurons following CNS insult [114, 115]. Converging lines of evidence now indicate that sigma-1 receptor activation may stimulate these GDNF-dependent repair mechanisms. In a spinal root avulsion model, daily administration of the sigma-1 agonist PRE084 after axotomization of motor neurons increased motor neuron survival at day 21 post-operation [108]. This recovery was accompanied by an early increase in GDNF expression in astrocytes in the ventral horn day 3 post-operation [108], suggesting that the activation of sigma-1 receptors in glial cells led to the release of survival-promoting trophic factors. The restorative effects to behavior and histology associated with subchronic treatment of PRE084 in the mouse model of PD described above was also accompanied by an upregulation of striatal GDNF and BDNF; GDNF was additionally upregulated in the substantia nigra [27]. Since phosphorylated ERK1/2 and protein kinase B were also increased under these conditions, the data suggest that downstream signaling pathways associated with these trophic factors were activated by PRE084 to promote recovery [27].

In addition, in a mouse model of ALS, subchronic treatment with PRE084 upon symptom onset increased BDNF immunoreactivity in the affected area: the ventral horn of the spinal cord in neurons and notably also in non-neuronal cells [26]. Confirmation that the upregulation of BDNF was mediated through sigma-1 receptors via pharmacological antagonism or genetic manipulation (knockdown or knockout) has yet to be conducted in the ALS model and represents an important future study.

In vitro findings further support a role for sigma-1 receptor activity in regulating BDNF. Heat-induced aggregation of BDNF and GDNF were blocked by purified sigma-1 polypeptides [116]. In addition, the sigma-1 agonist SA4503 stimulated mature BDNF secretion from SH-SY5Y (human neuroblastoma) and B104 (rat neuroblastoma) cells, an effect that could be prevented with the sigma-1 antagonist NE100 [114]. Knockdown of sigma-1 receptors in B104 cells also decreased the ability of the cells to secrete mature BDNF, further underscoring a potential role for sigma-1 receptors in regulating BDNF processing and release [116].

Although not studied in neurodegeneration models, sigma-1 agonists have also been reported to stimulate nerve growth factor (NGF)-induced neurite outgrowth in cultured cells [117–121] and potentiate epidermal growth factor (EGF)-induced neuritogenesis in PC12 (rat pheochromocytoma) cells overexpressing sigma-1 receptors [122]. In addition, sigma-1 activation with PRE084 enhanced neurite outgrowth in cerebellar granule cells via tropomyosin receptor kinase B (TrkB) signaling [123]. These observations raise the possibility that sigma-1 receptor activation has the potential to stimulate the activity of an array of neurotrophic factors to assist in recovery from CNS injury and disease.

10.4.2 Alterations in Neuronal Morphology

In many neurodegenerative conditions, aberrant neuronal morphology is observed. Dendritic and axonal deficiencies, in particular, are expected to compromise the integrity of neuronal connectivity within the CNS [124]. It is therefore noteworthy that sigma-1 receptors are found in key locations within neurons such as the growth cones [125], and agonists can promote neurite outgrowth from these sites through interactions with neurotrophic signaling pathways, as mentioned above. Additional studies have reported that decreased sigma-1 receptor expression can adversely affect dendritic arborization and axonal elongation in *in vitro* systems.

In hippocampal neurons, knockdown of sigma-1 receptors decreased dendritic arborization, diminished the formation and maturation of dendritic spines, and reduced protein markers of functional synapses [126]. Active forms of GTP (guanosine triphosphate)-binding Rac1 (ras-related C3 botulinum toxin substrate 1) and

intact TIAM1 (T-cell lymphoma invasion and metastasis-inducing protein 1) in raft fractions were also reduced in sigma-1 receptor knockdown neurons [126], suggesting the contribution of this signaling pathway to the decreased dendritic arborization. The ability of a constitutively active Rac or caspase-3-resistant TIAM1 construct to rescue spine formation in sigma-1 knockdown neurons was supportive of such a role [126]. In addition, a free radical scavenger (N-acetylcysteine), superoxide dismutase activator (Tempol), or NOS inhibitor (nitro-L-arginine) was able to restore spine formation in sigma-1 receptor knockdown neurons [126]. Together, the data suggest that deficits in sigma-1 receptors can compromise dendritic spine formation and arborization through a free radical-sensitive mechanism involving the Rac1-GTP pathway [126].

Another study recently demonstrated that sigma-1 receptor depletion or ablation can also compromise axonal morphology. Sigma-1 receptor knockout mice exhibited lower densities of axons, as measured by actin neurofilament immunostaining in the cortex, when compared to wildtype mice [127]. In addition, a slower rate of degradation of p35 was observed when sigma-1 receptors were depleted by knockdown *in vitro* or knockout *in vivo* [127]. P35 is a major activator of cyclin-dependent kinase 5 (cdk5), which plays an important role in cytoskeletal dynamics of microtubules and actin neurofilaments [128]. In contrast, overexpression of sigma-1 receptors in CHO cells resulted in a faster rate of degradation of p35 [127]. The influence of sigma-1 receptors on p35 appears to involve indirect interactions since direct physical interactions were not detected [127]. Notably, myristate was shown to bind to sigma-1 receptors as a putative agonist, resulting in increased phosphorylation of actin neurofilament proteins and myristoylation of p35 within 24 h. This modification of p35 increases its susceptibility to protein degradation [129], thus ultimately eliciting axonal extension in wildtype neurons and rescuing deficits in axonal elongation in sigma-1 knockdown neurons [127]. Together, the data suggest that sigma-1 receptors, in response to stimulation by myristic acid, can influence axonal elongation by modulating p35 turnover.

Collectively, the data suggest that therapeutic interventions that restore sigma-1 receptor expression or stimulate its function can reverse alterations in neuronal structure or morphology that result from or are associated with disease. In this regard, it is noteworthy that administration of the sigma-1 agonist PRE084 restored the deficits in sigma-1 receptor expression in a cellular model of HD, which were accompanied by reductions in a multitude of neurodegenerative markers (Hyrskyluoto et al. 2013). Future investigations to characterize the relationship between sigma-1 receptors and morphological changes in the context of neurodegenerative disease models and treatment, particularly under *in vivo* conditions, would be of value.

10.4.3 Recruitment of New Cells to Damaged Area

Damage to the nervous system is characterized by the loss of neurons, as well as the recruitment of glial cells to the site of injury. In response to CNS injury, sigma-1 agonists have been reported to enhance microglial and astrocytic activities that are associated with repair (see Sects. 10.3.5 and 10.3.6, respectively). Although not yet studied in the context of neurodegeneration, there is some evidence from depression models that sigma-1 agonists promote neurogenesis. For example, the selective sigma-1 agonist SA4503 promoted neurogenesis following subchronic treatment in stress-naive rats [130] and also in calcium/calmodulin-dependent protein kinase IV null mice exhibiting depressive behaviors and impaired neurogenesis [131]. In another animal model of depression involving olfactory bulbectomized mice, sigma-1 active compounds such as dehydroepiandrosterone (DHEA) similarly enhanced neurogenesis [132], an effect that was inhibited by treatment with the sigma-1 antagonist NE-100. In contrast, knockout of sigma-1 receptors in mice suppressed neurite growth and the survival of newborn neuronal

cells in the hippocampal dentate gyrus of adult mice [133]. Finally, stem cells which are under investigation for transplantation procedures [134] are enriched in sigma receptors [135].

10.5 Sigma-1 Receptor Activity as a Signal Amplifier in Neurodegeneration and Neuroprotection

Sigma ligands confer protective effects against many pathological mechanisms of neurodegeneration in preclinical studies, and have yielded great success as neuroprotective agents in different animal models of neurodegenerative disorders. However, the Food and Drug Administration (FDA) has yet to approve a selective sigma ligand for use in humans. In the single clinical study that tested a selective sigma ligand for the treatment of neurodegeneration, the selective sigma-1 agonist SA4503 failed to elicit significant functional recovery in the treated population compared to the placebo control after ischemic stroke [136]. However, post-hoc analysis of moderately and severely affected subjects showed significantly greater National Institutes of Health Stroke Scale improvements in the SA5403-treated group when compared with placebo ($P=0.034$ and $P=0.038$, respectively) [136]. Further clinical trials will be needed to optimize patient characteristics to identify a potential responder population, determine appropriate timing for treatment initiation and treatment duration, and evaluate the potential interaction of sigma-1 receptor therapy with other existing conventional pharmacological or non-pharmacological therapies.

It is noteworthy that many currently marketed psychotropic medications have significant affinity for sigma-1 receptors. Whether the therapeutic effects of these medications are mediated by sigma-1 receptor activity in humans remains unclear. Preclinical studies have shown that compounds such as fluvoxamine, DHEA-sulfate (DHEAS) and donepezil do elicit neuroprotective effects in part through activation of sigma-1 receptors, as their effects were attenuated with selective sigma-1 antagonists [13, 28, 137]. Below, we offer the hypothesis that alterations in the function or expression of sigma-1 receptors by themselves will likely be insufficient to cause disease or mitigate neurodegeneration. Rather, sigma-1 receptor dysfunction is likely to worsen disease progression, whereas stimulation may amplify pre-existing functional mechanisms of neuroprotection and/or restoration to slow down the disease progression.

10.5.1 Aberrant Sigma-1 Expression/Structure and Pathogenesis of Neurodegenerative Disease

Several recent studies have shown that decreased expression of sigma-1 receptors may contribute to the pathogenesis of neurodegenerative diseases. Mishina and colleagues, for example, used positron emission tomography (PET) with [^{11}C]SA4530 to demonstrate reduced densities of sigma-1 receptors in the frontal, temporal and occipital lobes, cerebellum, and thalamus of early AD patients [138]. A later study in PD patients showed that the binding potential of the PET ligand [^{11}C]SA4503 to sigma-1 receptors was significantly lower on the more affected than the less affected side of the anterior putamen [139]. However, there was no significant difference with respect to the binding potential between the patients and controls [139]. This supports the model that dysfunction in sigma-1 receptor protein expression augments, rather than initiates, disease progression.

Additionally, mutations in the sigma-1 receptor gene have been reported in ALS [93, 94], and sigma-1 receptor accumulates in intracellular protein aggregates in various neurodegenerative disorders, including trans-activation response DNA protein 43 proteinopathy, tauopathy, alpha-synucleinopathy, polyglutamine disease and intranuclear inclusion body disease [82, 140]. Since sigma-1 receptors have chaperone and regulatory roles [76, 84, 141], this accumulation may reflect a failed adaptive response to clear the inclusions during the course of the various diseases. It remains to be determined, however, whether these accumulations of sigma-1 recep-

tors represent accumulations of unfolded/non-functional or functional sigma-1 receptors. This accumulation may also contribute to disease progression by limiting the number of soluble sigma-1 receptors present in the cell, which may in turn potentiate ER stress and subsequent apoptosis. This suggests that sigma-1 receptor dysfunction is a later effect in the pathologic process, after neurodegeneration has begun but possibly before the manifestation in clinical symptoms. Targeting the remaining functional sigma-1 receptors with sigma-1 agonists may therefore slow down disease progression (see Sect. 10.5.2).

Evidence from sigma-1 receptor knockdown and knockout studies in cellular and animal studies further support this hypothesized contributory role of sigma-1 receptors. In CHO cells, the knockdown of sigma-1 receptors destabilized the conformation of IRE1 and decreased cell survival following administration of the ER stressor thapsigargin [84]. In contrast, under vehicle conditions (in the absence of thapsigargin), sigma-1 knockdown had no effect on the stability of IRE1 or apoptosis [84]. In animal studies, Langa and colleagues demonstrated that homozygous mutant mice (mouse sigma-1 receptor gene, *mSR1−/−*) were viable and fertile with negligible overt phenotype compared with their wildtype littermates [142]. Mavlyutov et al. found that knockout of sigma-1 receptors produced slight motor abnormalities on a rotarod test, but did not itself result in an ALS phenotype or increased weight loss [143]. On the other hand, knockout of sigma-1 receptors in a SOD1-G93A mouse model of ALS exacerbated weight loss, produced an early decline in swimming performance, and ultimately decreased longevity [25].

Supporting the hypothesis that the deleterious consequences of sigma-1 receptor malfunction or aberrant expression manifest primarily under conditions of stress, retinal development also appears normal in sigma-1 knockout mice, with significant deficits (e.g., increased RGC loss and increased intraocular pressure) observed only with advanced age [144, 145]. A recent study showed that RGC death is accelerated in sigma-1 receptor knockout mice compared to wildtype following optic nerve crush, a model system for triggering apoptotic responses similar to those seen in glaucoma [144]. More extensive characterization has also been performed in sigma-1 receptor knockout mice with streptozotocin (STZ)-induced diabetes. Similar to the ocular crush model, STZ treatment accelerated retinal damage in sigma-1 receptor knockout mice; diabetic sigma-1 knockout mice showed fewer RGCs and more caspase-3 positive cells compared to non-diabetic wildtype mice, while sigma-1 knockout alone had no effects [145]. Additionally, relative to the other groups tested (non-diabetic knockout, non-diabetic wildtype and diabetic wildtype), diabetic sigma-1 receptor knockout mice showed increased intraocular pressure and deficits in scotopic threshold responses, which are the most sensitive electroretinogram responses observable with dim stimuli in the dark-adapted state and reflect RGC health [145].

10.5.2 Sigma-1 Activation as Intracellular Amplifier of Pre-existing Neuroprotective Mechanisms

Su and Hayashi proposed that sigma-1 receptors act as intracellular amplifiers for signal transduction, describing the biochemical actions of sigma-1 receptors as modulatory in nature and that the functional implications of these receptors may only be manifested when another biological system is first activated [146]. Consistent with this, the selective sigma-1 agonist (+)-pentazocine prolonged the association of sigma-1 receptors with IP_3 receptors under ER stress but had no effect under normal conditions [76]. Likewise, sigma-1 agonists, without effects by themselves, potentiated bradykinin-induced alterations in cytosolic free Ca^{+2} concentrations [147]. In addition, Monnet and colleagues showed that in anesthetized rats, (+)-pentazocine had no detectable effects on its own, but potentiated NMDA-mediated glutamatergic stimulation [148, 149].

Relevant to neurodegenerative diseases, abnormal intracellular sigma-1 protein aggregates have been reported in various disorders, as

mentioned in Sect. 10.5.1. Specific to ALS, sigma-1 receptor accumulation has been observed in lumbar alpha motor neurons of ALS patients and SOD1-G93A mice, cultured fibroblasts from ALS-8 patients with the P56S-VABP (vesicle-associated membrane protein-associated protein B) mutation, and in NSC34 (mouse motor neuron-like hybrid) cells transfected with the P56S-VABP mutation [140]. These accumulations co-localized with VAPB in the fibroblasts and NSC34 cells with the P56S-VABP mutation [140]. VABP is another ER protein, in which the P56S point mutation causes severe misfolding of the peptide and leads to the formation of cytoplasmic inclusion bodies and familial ALS [140]. Importantly, activation of sigma-1 receptors by PRE084 in P56S-VABP NSC34 cells ameliorated mutant VAPB aggregation and increased the degradation of soluble mutant VAPB without affecting the normal level of the wildtype proteins [140]. These results suggest targeting sigma-1 receptors with agonists can help ameliorate protein aggregation and inhibit disease progression by enhancing their innate chaperone activity.

Relevant to the neurorestorative potential of sigma-1 receptors, in PC12 cells, several sigma-1 agonists including (+)-pentazocine, imipramine, fluvoxamine and donepezil showed no effects on their own but potentiated NGF-induced neurite outgrowth [117–120]. Co-administration of the sigma-1 antagonist NE-100 blocked this effect, confirming the specificity of sigma-1 receptor involvement [117–120]. Moreover, the overexpression of sigma-1 receptors enhanced the NGF-induced neurite sprouting, while antisense deoxyoligonucleotides directed against sigma-1 receptors attenuated the NGF-induced neurite outgrowth [120].

10.5.3 Proposed Use of Sigma-1 Ligands in a Multi-target Therapeutic Approach

Due to the intrinsic modulatory role of sigma-1 receptors in disease and therapy, sigma-1 receptor activation as a stand-alone treatment appears unlikely to be sufficient to elicit observable clinical outcomes. The large body of preclinical evidence using primarily selective sigma-1 compounds described above indicates sigma-1 receptors are viable targets for therapeutic applications for CNS-related disorders. However, to combat the complex and multi-dimensional nature of neurodegenerative diseases, a multi-treatment approach would likely be most beneficial. Sigma-1 ligands, with the ability to affect multiple mechanisms and neural cell types that contribute to neurodegeneration through sigma-1 receptor activation, may therefore offer greater promise as an adjunct therapy. As we mentioned above, though there are no currently approved selective sigma-1 compounds for use in humans, many currently available psychotropic drugs interact with sigma-1 receptors. Using PET with [^{11}C]SA4503, Ishikawa and colleagues showed that fluvoxamine, which has the highest affinity for sigma-1 receptors among SSRIs, binds to sigma-1 receptors in living human brains at therapeutic doses [150]. A follow-up study also showed that the acetylcholinesterase inhibitor donepezil binds to sigma-1 receptors at therapeutic doses [151]. Recently, in a mouse model of amyloid beta (25–35)-induced memory impairments, Maurice showed that protection by the sigma-1 agonist PRE084 is synergistic with donepezil [152]. Therefore, the repurposing or development of sigma-1 receptor active drugs, selective or not, requires further investigation as viable therapeutic approaches for treating neurodegenerative diseases. Moreover, the usage of selective sigma-1 ligands as an adjunct (vs. standalone) treatment may prove more fruitful in clinical trials and serve to validate the potential therapeutic significance of sigma-1 receptors as amplifiers of neuroprotective actions.

10.6 Conclusion

Sigma-1 receptors, with their wide range of effects on multiple signaling pathways, appear to be promising, druggable targets to help combat the complex pathophysiology of neurodegenerative disorders. In its apparent role as an intracel-

lular amplifier, however, sigma-1 receptor activation will likely be most effective in a multi-target therapeutic approach in conjunction with other pharmacological interventions. Further understanding the signaling cascades regulated by sigma-1 receptors will aid in the development of novel therapies to slow the progression of neurodegeneration and/or reverse existing pathologies.

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11 Sigma-1 Receptor Agonists and Their Clinical Implications in Neuropsychiatric Disorders

Yakup Albayrak and Kenji Hashimoto

Abstract

Accumulating evidence suggests that sigma-1 receptors play a role in the pathophysiology of neuropsychiatric diseases, as well as in the mechanisms of some selective serotonin reuptake inhibitors (SSRIs). Among the SSRIs, the order of affinity for sigma-1 receptors is as follows: fluvoxamine > sertraline > fluoxetine > escitalopram > citalopram >> paroxetine. Some SSRIs (e.g., fluvoxamine, fluoxetine and escitalopram) and other drugs (donepezil, ifenprodil, dehydroepiandeterone (DHEA)) potentiate nerve-growth factor (NGF)-induced neurite outgrowth in PC12 cells, and these effects could be antagonized by the selective sigma-1 receptor antagonist NE-100. Furthermore, fluvoxamine, donepezil, and DHEA, but not paroxetine or sertraline, improved phencyclidine-induced cognitive deficits in mice, and these effects could be antagonized by NE-100. Several clinical studies showed that sigma-1 receptor agonists such as fluvoxamine and ifenprodil could have beneficial effects in patients with neuropsychiatric disorders. In this chapter, the authors will discuss the role of sigma-1 receptors in the mechanistic action of some SSRIs, donepezil, neurosteroids, and ifenprodil, and the clinical implications for sigma-1 receptor agonists.

Keywords

Donepezil • Ifenprodil • Fluvoxamine • Psychiatric diseases

11.1 Introduction

The history of discovery of sigma receptors has existed for 40 years. These receptors were firstly considered to be associated with opioid receptors and accepted as a subtype of opioid receptors [1]. In the following years, sigma receptors were

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thought to contribute to N-methyl-D-aspartate (NMDA) subtype of glutamate receptors. After the misidentifications of sigma-1 receptors that mentioned above, there has been better understanding of effects of sigma receptors in nervous system and as well as other systems for the past 15 years in both experimental and clinical manners [2, 3]. Currently, sigma receptors have been confirmed as a non-opioid, endoplasmic reticulum (ER) protein and they have been subclassified as sigma-1 and sigma-2 receptors according to their action.

11.2 General Information About Sigma 1 Receptors

After the subclassification of sigma receptors as sigma-1 and sigma-2, sigma-1 receptor has been successfully cloned as a 223 amino acid protein with two transmembrane domains in 1996 [4]. In 2007, sigma-1 receptor was described as a ligand-responsive ER chaperone protein [5]. Sigma-1 receptor is predominantly expressed at the ER membrane and has 24-kDa molecular mass [6–8]. The C-terminus of these receptors contain molecular chaperone activity [5]. The sigma-1 receptors are unique, and they are not similar with other mammalian molecular chaperone molecules [9, 10]. The role of molecular chaperones at the ER is to promote suitable folding of new synthesized proteins [11]. These molecules modulate misfolded proteins. Thus, the action of chaperone molecules is quite important to protect toxic protein accumulations and lead to cellular survival during stressful conditions [11]. There has been a strong link between regulation of chaperone activity of sigma-1 receptors and another ER chaperone immunoglobulin heavy chain binding protein (Bip)/GRP78 [5, 10]. When the sigma-1 receptors come together with BiP, the chaperone activity becomes minimum. If sigma-1 receptors separate from BiP, the capacity of the chaperone becomes maximum. It is well established that several synthetic agents, which have the property of sigma-1 receptor agonism, lead to the dissociation of sigma-1 receptors from BiP and enhance the chaperone activity. In contrast, the agents with sigma-1 receptor antagonism reinforce the linkage between two proteins and minimize the chaperone activity [5, 9, 10].

The sigma-1 receptors are the first molecular chaperones whose activities can be regulated with agonist and antagonist agents [12]. They have a unique character and thus, by their chaperone activity, novel pharmacological agents can alter the accumulation of misfolded proteins. There are a number of agents with sigma-1 receptor agonistic activity, and they could be novel and promising therapeutic agents for a number of diseases [12].

Regarding the sigma-1 receptor chaperone activity, it has been reported to have effects on numerous cellular functions such as Ca^{+2} signaling, ion channel activities, cellular redoxing, neurotransmitters, cellular survival, neurotransmitters , synaptogenesis and inflammation [2, 3, 12, 13]. Preclinical and clinical studies demonstrated that sigma-1 receptors can affect progression of cardiovascular diseases, immune reactions, cancer proliferation, pain, liver functions and various neuropsychiatric diseases [8, 12, 14–18].

11.3 Sigma 1 Receptor Agonists in Neuropsychiatric Diseases

Sigma-1 receptors do not only exist in central nervous system (CNS), but also exist in peripheral organs such as liver and spleen. Here, we focused on the sigma-1 receptor agonists, which play a role in neuropsychiatric diseases.

11.3.1 Donepezil

Donepezil is a commonly used drug, which plays an important role in the management of Alzheimer's Disease (AD). Donepezil inhibits acetylcholinesterase (AChE) and enhances the cognitive functions as well as general functionality that is disturbed during the disease. Donepezil not only inhibits AChE, it also binds to sigma-1 receptor in the brain [19]. Ishima et al. [20] reported that donepezil significantly enhanced nerve growth factor (NGF)-induced neurite

outgrowth in PC12 cells, and that sigma-1 receptor antagonist NE-100 blocked its effect. Moreover, donepezil could improve phencyclidine (PCP)-induced cognitive deficits, and its effects were also antagonized by treatment with NE-100 [19]. Positron emission tomography (PET) studies showed that donepezil bound to sigma-1 receptors in human brain after a single oral administration [21]. Taken together, sigma-1 receptor chaperone activity of donepezil might be involved in the mechanism of action at therapeutic doses.

11.3.2 Neurosteroids

Neurosteroids are produced from cholesterol in the CNS as well as in peripheral nervous systems. Neurosteroids –specifically progesterone- are known to bind sigma receptors in brain and spleen. There are associations between immune, endocrine and nervous system via neurosteroids [22, 23]. Dehydroepiandrosterone (DHEA) is the most abundant neurosteroid that has a moderate sigma −1 receptor agonistic activity (Ki= 706 nM), and progesterone and testosterone are known to be sigma-1 receptor antagonists (Ki= 268 or 36 nM and Ki= 1014 or 201 nM; respectively) [16]. Thus, it is likely that endogenous neurosteroids bind to sigma-1 receptors in CNS and peripheral nervous system. DHEA-sulfate (DHEA-S) could improve PCP-induced cognitive deficits in mice, and its effect was antagonized by treatment with NE-100 [24]. İt is suggested that neurosteroids such as DHEA-S have neuroprotective effects via sigma-1 receptor agonism.

11.3.3 Ifenprodil

Ifenprodil is a cerebral vasodilator agent that is used in a limited numbers of countries. The mechanism of action of ifenprodil is the antagonism of GluN2B subtype of NMDA receptor. Beside NMDA receptor antagonism, ifenprodil binds to sigma-1 and sigma-2 receptors in the brain [25–27]. Ifenprodil could enhance NGF-induced neurite outgrowth in PC12 cells, and its effect was antagonized by NE-100, but not sigma-2 receptor antagonist SM-21 [28]. Taken together, it is likely that ifenprodil is a potent sigma-1 receptor agonist.

There are some articles showing beneficial effects of ifenprodil in patients with neuropsychiatric disorders. Ifenprodil was effective in the treatment of flashbacks in three patients with post-traumatic stress disorder (PTSD) who had a childhood sexual abuse [29]. Subsequently, Sasaki et al. [30] also reported that ifenprodil was effective in the treatment of flashbacks in three female PTSD patients with a childhood abuse. These case reports suggest that ifenprodil therapy could be an alternative treatment for flashbacks in adult and adolescent PTSD patients with a childhood abuse [31] although the precise mechanisms underlying its mechanisms are currently unknown. A randomized, double-blind placebo-controlled study of ifenprodil in PTSD patients is underway at Chiba University Hospital (NCT01896388).

11.3.4 Selective Serotonin Reuptake Inhibitors

Selective serotonin reuptake inhibitors (SSRIs) are commonly used in the management and treatment of major depressive disorder (MDD), anxiety disorders, obsessive compulsive disorder (OCD), and other neuropsychiatric disorders. The mechanism of action of SSRIs is commonly described as blocking serotonin reuptake and so elevating the level of serotonin in synaptic area [32–34]. Although all SSRIs are considered to share a similar mechanism, their beneficial effects and side effects differ from patient to patient. Although this discrepancy of effects and side effects are suggested to be their pharmacokinetic and pharmacodynamic properties, the interactions of SSRIs at sigma-1 receptor might be involved. Some SSRIs have been shown to have moderate to high affinity to sigma-1 receptors, but not sigma-2 receptors [35, 36] (Table 11.1). The order of potency for SSRIs at sigma-1 receptor chaperones is as follows: fluvoxamine (Ki = 17.0 nM) >

Table 11.1 Pharmacology of SSRIs at sigma-1 receptors in rat brains

Drugs	Ki (nM)	Pharmacology
Fluvoxamine	17	Agonist
Sertraline	31.6	Antagonist
Fluoxetine	191.2	Agonist
Escitalopram	288.3	Agonist
Citalopram	403.8	Agonist
Paroxetine	2041	–

From Ref. [36]

sertraline (Ki = 31.6 nM) > fluoxetine (Ki = 191.2 nM) > escitalopram (Ki = 288.3 nM) > citalopram (Ki = 403.8 nM) >> paroxetine (Ki = 2041 nM) [36]. In contrast, serotonin and norepinephrine reuptake inhibitors (SNRIs) have no affinity at sigma-1 receptors [36]. A study using PET showed that orally administered fluvoxamine bound to sigma-1 receptors in the intact human brain [37]. These findings suggest that sigma-1 receptors might play a role in the mechanistic action of SSRIs, such as fluvoxamine, fluoxetine and escitalopram.

11.3.5 Sigma-1 Receptor Agonists in Cognition

Cognitive impairments are shown in patients with a number of neuropsychiatric disorders such as MDD (major depressive disorder), schizophrenia, dementia and delirium. In MDD patients, cognitive, psychomotor and memory impairments are seen commonly [38–42].

Sigma-1 receptors can be associated with various neurotransmitter system and can affect their functions [8]. Some SSRIs such as fluvoxamine, fluoxetine and escitalopram could enhance NGF-induced neurite growth in PC12 cells via sigma-1 receptor activation [43–45]. In contrast, another SSRI, sertraline, with a high affinity at sigma-1 receptors did not affect NGF-induced neurite outgrowth [36, 44]. Interestingly, sertraline could antagonize the effect of fluvoxamine, suggesting that sertraline may be a sigma-1 receptor antagonist. PCP-induced cognitive deficits were improved by subsequent repeated administration of fluvoxamine, but not sertraline [24, 45].

In a randomized, double-blind study, fluvoxamine was found to increase performance of digit symbol substitution test [46]. Four weeks of fluvoxamine treatment was reported to be related with higher total Wechsler IQ scores beside the significant improvement in symptoms in MDD patients. Furthermore, there was also lower incidence of cognitive impairment in patients who responded to fluvoxamine treatment [47]. In two case studies, fluvoxamine was reported to improve cognitive functions in patients with schizophrenia [48, 49]. After these two case reports, a randomized, double-blind, placebo controlled study was performed to investigate the effects of fluvoxamine on cognitive functions in medicated patients with schizophrenia. Although fluvoxamine does not have a major impact on the cognitive impairments in patients with schizophrenia, secondary analysis showed only improvement in executive functions [50, 51]. These findings highlight the need for further large scale studies to confirm the effects of SSRIs with sigma-1 receptor activity on cognitive impairment.

11.3.6 Sigma-1 Receptor Agonists in Psychotic Depression

Psychotic depression is a subtype of MDD in which psychotic symptoms, more severe depressive symptoms, suicidal ideation and attempts, psychomotor retardation and severe cognitive impairment occur. For the treatment of psychotic depression, a combination of antidepressants and antipsychotics or electroconvulsive therapy are the most acceptable treatment choices. However, these treatments can cause some severe side effects such as extrapyramidal side effects (EPS) and cognitive impairment [52]. Interestingly, fluvoxamine was reported to improve both psychotic and depressive symptoms [53, 54]. In contrast, paroxetine has been found to have lesser effects on psychotic symptoms [55]. It has been suggested that the beneficial effects of fluvoxamine on symptoms of psychotic depression via both serotonin reuptake inhibition and sigma-1 receptor agonism [56, 57]. Subsequently, Furuse

and Hashimoto [58] reported five cases with psychotic depression who had benefited effectively from fluvoxamine monotherapy. Kishimoto et al. [59] also reported an interesting case with psychotic depression whose symptoms worsened switching from fluvoxamine to sertraline. Furthermore, the symptoms of patients disappeared after fluvoxamine 150 mg/day treatment. From the results of this study, it has been concluded that sigma-1 receptor agonism by fluvoxamine might be implicated in the mechanism of action [18, 56, 57, 60]. However, further detailed clinical studies are needed to confirm the beneficial effects of fluvoxamine on psychotic depression via sigma-1 receptor activity.

11.3.7 Sigma-1 Receptor Agonists in Delirium

Delirium is a clinical situation, which includes impairment of consciousness and cognition, hallucinations, abnormality in psychomotor state, and sleep-wake disturbances. It can be seen in elderly patients, chronic medical conditions and after surgical interventions. It can be considered as a worse prognosis among inpatients and patients who have chronic or end-stage diseases. The exact pathophysiology of delirium is unknown; however neurotransmitters that include dopamine, serotonin, norepinephrine, acetylcholine, glutamate, γ-aminobutyric acid are considered to be related to development of delirium [61, 62].

Both typical and atypical antipsychotics have been used for symptomatologic treatment of delirium. Because the side effects of antipsychotics such as extrapyramidal side effects, akathisia, dystonia, hypotension, laryngeal spasms, anticholinergic side effects such as constipation, dry mouth and urinary retention, neuroleptic malignant syndrome, metabolic side effects such as glucose and lipid dysregulation, patients with delirium who received these agents should be monitored closely [61–64]. Furthermore, atypical antipsychotics were reported to be related with sudden deaths in patients with dementia. In treatment of delirium, which is associated with sedative-hypnotic withdrawal, benzodiazepines are the first choice [65].

There are some papers showing the association between SSRIs and delirium. Among SSRIs, sertraline and paroxetine were reported to be associated with development of delirium in combination treatments [66, 67]. It has been reported that citalopram (20 mg i.v.) treatment exhibited delirium after 3 days. There were also case studies that SNRIs such as duloxetine and venlafaxine are associated delirium [68–70].

In contrast, fluvoxamine was reported to have beneficial effects on delirium. Furuse and Hashimoto reported that fluvoxamine was effective in the treatment of delirium in patients with Alzheimer’s disease [71], patients in intensive care units [72], and elderly patients with postoperative delirium [73]. Regarding the case studies which reported the beneficial effects of fluvoxamine on delirium symptoms, it can be considered that fluvoxamine might be a promising alternative agent for treating the symptoms of delirium without serious side effects. The mechanism of action of fluvoxamine for symptoms of delirium may be due to sigma-1 receptor agonism [74].

11.3.8 Sigma-1 Receptor Agonists for Prevention of Psychosis

Schizophrenia is a chronic, disabling psychiatric disorder, affecting almost 1 % of the world’s population. The symptoms of schizophrenia include positive and negative symptoms, cognitive impairment, and a deterioration in social and occupational functioning [75]. Prodromal symptoms of schizophrenia are considerable in terms of decreasing the risk of development schizophrenia [76, 77]. Cognitive impairment is one of the most reliable indicators of prodromal symptoms for predicting schizophrenia [78]. Considering the beneficial effects of sigma-1 receptor agonist fluvoxamine on cognitive impairments [51], fluvoxamine may have beneficial effects on prodromal symptoms [79]. Based on this hypothesis, Takodoro et al. [80] presented a case report showing that fluvoxamine might

prevent the onset of psychosis in a high-risk subject. However, further detailed studies using a large sample are needed to support this hypothesis.

11.3.9 Sigma-1 Receptor Agonists for Akathisia and Other Hyperkinetic Movement Disorders

Akathisia is a side effect, which is characterized with an inability to sit or remain motionless [81]. Akathisia is commonly regarded as a side effect of antipsychotics –specifically typical ones- and can cause suicide attempts [82]. β-Adrenergic blockers, benzodiazepines and anticholinergics are commonly used in the management of akathisia [83]. Sigma-1 receptor agonist fluvoxamine was firstly reported to be effective in the treatment of akathisia by Furuse and Hashimoto [84, 85]. We also reported a patient with tardive akathisia case who improved with fluvoxamine over a short time period [86]. Regarding the unclear etiology of akathisia, fluvoxamine may be effective and an alternative treatment choice for akathisia by sigma-1 activation [8, 11, 15, 40].

Sigma-1 receptors are known to play a role in the neurocircuits of movement [87]. There are small numbers of papers showing the possible effects of sigma-1 receptor agonists on movement disorders. We reported on three patients with schizophrenia with tardive dyskinesia, and fluvoxamine achieved a gradual disappearing of symptoms [86]. We also reported five cases with post-psychotic depressive disorder and schizophrenia, where depression and tardive dyskinesia was improved with fluvoxamine [88]. Additionally, a patient who developed tardive dyskinesia during duloxetine treatment was reported to benefit from switching to fluvoxamine [89]. In other case studies, improvements in chorea and hemiballism in patients with Huntington's disease and depression were reported [90, 91]. Regarding the reports mentioned above, fluvoxamine may be a promising treatment of choice in hyperkinetic movement disorders. However, further studies are needed.

11.4 Conclusion

Given the molecular chaperone activity of sigma-1 receptors, sigma-1 receptor agonists such as donepezil, ifenprodil and fluvoxamine, would be promising drugs for a number of neuropsychiatric disorders. However, further detailed clinical studies using a greater number of patients are needed to confirm the effects of sigma-1 receptor agonists on neuropsychiatric disorders.

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12 Role of Sigma-1 Receptor in Cocaine Abuse and Neurodegenerative Disease

Yu Cai, Lu Yang, Fang Niu, Ke Liao,
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Abstract

Sigma-1 receptors (Sig-1R) are recognized as a unique class of non-G protein-coupled intracellular protein. Sig-1R binds to its ligand such as cocaine, resulting in dissociation of Sig-1R from mitochondrion-associated ER membrane (MAM) to the endoplasmic reticulum (ER), plasma membrane, and nuclear membrane, regulating function of various proteins. Sig-1R has diverse roles in both physiological as well as in pathogenic processes. The disruption of Sig-1R pathways has been implicated as causative mechanism(s) in the development of both neurodegenerative disorders such as Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington Disease (HD). Additionally, the interaction of cocaine and Sig-1R has more recently been implicated in potentiating the pathogenesis of HIV-associated neurocognitive disorders (HAND) through impairment of blood-brain barrier (BBB), microglial activation and astrogliosis. On the other hand, restoration of Sig-1R homeostasis has been shown to exert neuroprotective effects. In this review, we provide an overview of how Sig-1R plays a role in the pathogenesis of neurodegenerative disorders and cocaine and implications for future development of therapeutic strategies.

Keywords

Sigma-1 receptor (Sig-1R) • Alzheimer Disease (AD) • Parkinson Disease (PD) • Amyotrophic Lateral Sclerosis (ALS) • Huntington Disease (HD) • Cocaine

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Abbreviations

6-OHDA	6-hydroxydopamine
ALCAM	activated leukocyte cell adhesion molecule
AD	Alzheimer Disease
Aβ	amyloid beta
ALS	Amyotrophic Lateral Sclerosis
APOE 4	apolipoprotein E gene
BIP	binding immunoglobulin protein
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
CHO cells	Chinese hamster ovary cells
CAG	cytosine-adenine-guanine
Egr-1	early growth response gene 1
ER	endoplasmic reticulum
FDA	Food and Drug Administration
FTLD	Frontotemporal Lobar Dementia
GDNF	glial cell line-derived neurotropic factor
GFAP	glial fibrillary acidic protein
HAND	HIV-associated neurocognitive disorders
HBMEC	human brain microvascular endothelial cells
HD	Huntington Disease
LID	levodopa-induced dyskinesia
MAM	mitochondrial-associated membrane
MAPK	mitogen-activated protein kinase
MNs	motor neurons
NSDUH	National Survey on Drug Use and Health
NO	nitric oxide
NF-κB	nuclear factor-κB
PD	Parkinson Disease
PDGF-BB	platelet-derived growth factor-BB
PDGF-β	platelet-derived growth factor-β
PET	positron emission tomography
RBMVEC	rat brain microvascular endothelial cells
FUS	RNA-binding protein Fused in Sarcoma
SETX	senataxin
Sig-1R	Sigma-1 receptor
Sig-2R	Sigma-2 receptor
SOCE	store-operated Ca2+ entry
SOD1	superoxide dismutase-1 protein
TARDBP	TAR DNA-binding protein 43

12.1 Introduction

Drug addiction involving the use of psychostimulants such as cocaine and methamphetamine is a growing epidemic globally. Data from National Survey on Drug Use and Health (NSDUH) showed that in 2011, 670,000 Americans aged 12 or older had used cocaine [1] . It has been well documented that chronic cocaine users often suffer from symptoms such as anxiety, depression, drug craving and cognitive impairment [2, 3]. Cocaine abuse in the elderly also poses serious challenge to the health care management in the United States. In fact, one study of inner city emergency department showed that 2 % of visiting patients aged over 60 years were cocaine positive based on the urine test [4]. Moreover, structural MRI brain scan studies have also shown that the reduction rate of global gray matter volume in the cortical and subcortical regions of chronic cocaine users was nearly twice the rate of healthy volunteers, suggesting accelerated ageing in cocaine-dependent individuals [5]. A vicious cycle thus manifests, in which cocaine users suffer from cognitive decline resulting from both cocaine abuse as well as ageing.

In addition to the mental health consequences of cocaine, increased incidence of neurodegenerative disorders is also a growing concern as individuals continue to live long. Neurodegenerative disorders, including Alzheimer Disease (AD), Parkinson Disease (PD), Amyotrophic Lateral Sclerosis (ALS) and Huntington Disease (HD), are characterized by progressive loss of neuronal functioning and numbers. These disorders are posing a major challenge for health care management with the global increase in aging population. AD is one of the most common neurodegenerative diseases, with 46.8 million people affected worldwide [6]. PD is the second common neurodegenerative condition, affecting around 2 % of the population above age of 65 [7]. The prevalence of ALS in United States is 3.9 cases per 100,000 general population, and it is more common among persons aged 60-69 years [8]. Approximately five to seven out of every 100,000 people are affected by

HD in Western countries [9]. It is therefore essential to discover novel approaches to alleviate cognitive impairment induced by cocaine and/or age-related neurological disorders.

Sigma receptors were formerly misclassified as a subtype of opioid receptor in 1970s. However, over the years their role as a unique class of non-G protein-coupled intracellular protein receptors that bind to cocaine has become more clear [10]. Sigma receptors have two subtypes, namely Sigma-1 receptor (Sig-1R) and Sigma-2 receptor (Sig-2R). Sig-2R is a universally distributed protein that modulates cell proliferation and tumor pathogenesis [11]. Sig-1R is an intracellular molecular chaperone (28 kDa) that predominantly resides in the endoplasmic reticulum (ER), especially ER sub-region contacting mitochondria, namely the mitochondrial-associated membrane (MAM) [12, 13]. Highly expressed in the brain, Sig-1R is also a transmembrane protein that regulates various cellular activities including synaptic plasticity, modulation of ion channels, ER stress, astrogliosis and microglia activation [14]. Recent studies have also shown that Sig-1R plays an important role in drug addiction especially cocaine abuse, psychiatric disorders and neurodegenerative disorders [15]. Understanding how Sig-1R plays a role in the pathogenesis of neurodegenerative disorders and cocaine abuse will be the subject of this review.

12.2 Role of Sigma-1 Receptors in Cocaine and HIV-Mediated Cognitive Impairment

Despite the advent of combination antiretroviral therapy (cART), there is increased prevalence of HIV-associated neurocognitive disorders (HAND) [16], as infected individuals continue to live longer. Almost 40-60 % of infected individuals on cART are known to suffer from some form of HAND. The key underlying correlate of HAND is inflammation, both in the periphery and in the CNS. Adding further layer of complexity to HAND is the increased abuse of drugs in those infected with HIV. Drugs of abuse further exacerbate neuroinflammation associated with HIV-1. Interestingly, one such drug - cocaine, has been shown to exacerbate neuroinflammation via several molecular pathways involving disruption of blood-brain barrier (BBB) integrity, astrogliosis, microgliosis and neuronal injury/death [17–22]. Sig-1R is known to play an essential role in cocaine-related neurologic effects. Previous studies have demonstrated that Sig-1R is localized in both the CNS and in the periphery where cocaine poses its toxic effects, and interacts with Sig-1R at physiologically relevant concentrations [23]. Cocaine acts as an agonist for Sig-1R that regulates dissociation of Sig-1R from ER chaperone binding immunoglobulin protein (BIP) [24, 25]. Dissociated Sig-1R translocates from mitochondrion-associated ER membrane (MAM) to the ER, plasma membrane, and nuclear membrane, regulating functions of various proteins [25]. Recent evidence has further indicated that cocaine-mediated translocation of Sig-1R resulted in increased vascular permeability [17], impairment of the blood-brain barrier (BBB) [18], microglial activation [19, 26], astrogliosis [20, 27] and neuronal injury [21, 28]. Herein we discuss the role of Sig-1R in response to cocaine/HIV proteins in various cells of the CNS.

12.2.1 Blood Brain Barrier (BBB)

Under normal conditions the BBB functions as a highly selective permeability barrier that regulates homeostasis of the CNS microenvironment. During injury or insult following exposure to noxious drugs, BBB can be breached rendering the brain vulnerable to infectious pathogens and toxins in the circulating blood [29]. There are extensive reports suggesting the role of Sig-1R in cocaine-mediated disruption of BBB through distinct intracellular mechanisms [17, 18, 30]. In fact one of the reports from our groups demonstrated that exposure of human brain endothelial cells (HBMEC) to cocaine resulted in increased permeability of these cells via the activation of Sig-1R, leading in turn, to upregulation of platelet-derived growth factor-BB (PDGF-BB) [17]. Detailed signaling pathways involved in this process involved sequential activation of

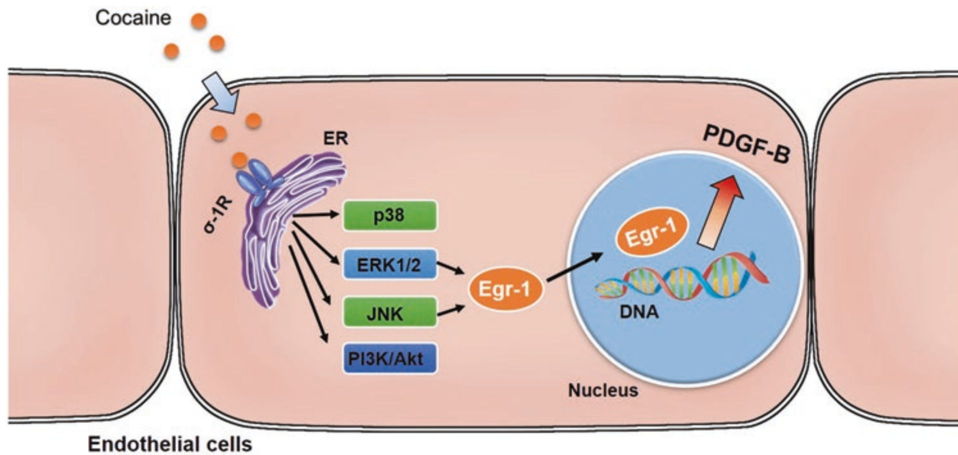

Fig. 12.1 Schematic of the molecular mechanisms involved in increased permeability of HBMECs induced by cocaine. Exposure of human brain endothelial cells (HBMEC) to cocaine resulted in increased permeability of these cells via the activation of Sig-1R, leading in turn, to upregulation of platelet-derived growth factor-BB (PDGF-BB). Detailed signaling pathway involved in this process involved sequential activation of mitogen-activated protein kinase (MAPK) and Egr-1 pathways

mitogen-activated protein kinase (MAPK) and Egr-1 pathways (Fig. 12.1). These findings were also validated *in vivo* wherein enhanced permeability in cocaine-treated mice could be abrogated by either pre-treating the mice with neutralizing antibody for PDGF-BB or using the Egr-1 knockout mice. Another possible mechanism for cocaine/Sig-1R-mediated endothelial dysfunction was also reported to function via inhibition of store-operated Ca^{2+} entry (SOCE) [30]. In this report it was shown that cocaine inhibited SOCE in rat brain microvascular endothelial cells (RBMVEC) and, this effect could be suppressed by both an antagonist and shRNA for Sig-1R. Additionally, our group was the first to demonstrate that cocaine-mediated translocation of Sig-1R to the plasma membrane resulted in the upregulation of activated leukocyte cell adhesion molecule (ALCAM) in the HBMEC, leading subsequently, to increased monocyte adhesion/transmigration, thereby resulting in exacerbated neuroinflammation [18]. Cocaine mediated induction of ALCAM involved translocation of Sig-1R to the plasma membrane, subsequent phosphorylation of PDGF-β (PDGF-β) followed by downstream activation of MAPK, Akt and NF-κB pathways (Fig. 12.2). Along these lines, we also observed that upregulation of ALCAM in the brain endothelium of HIV-infected cocaine users was accompanied by increased monocyte/macrophage infiltration when compared with HIV-positive individuals without drug abuse history or uninfected controls. These findings were further corroborated by the fact that neutralizing antibody to ALCAM ameliorated cocaine-mediated exacerbation of monocyte adhesion and transmigration *in vitro*. Understanding the regulation and functional changes of BBB by cocaine/Sig-1R axis could provide insights into the development of potential therapeutic targets for HAND.

12.2.2 Microglia

Both migration and activation of microglia/macrophages play an important role in pathogenesis of neurological disorders such as HAND. Using pharmacological inhibitors, pioneering study has indicated the involvement of both Sig-1R and TGF- β in upregulation of HIV-1 expression in microglial cell cultures in vitro [31]. Previous

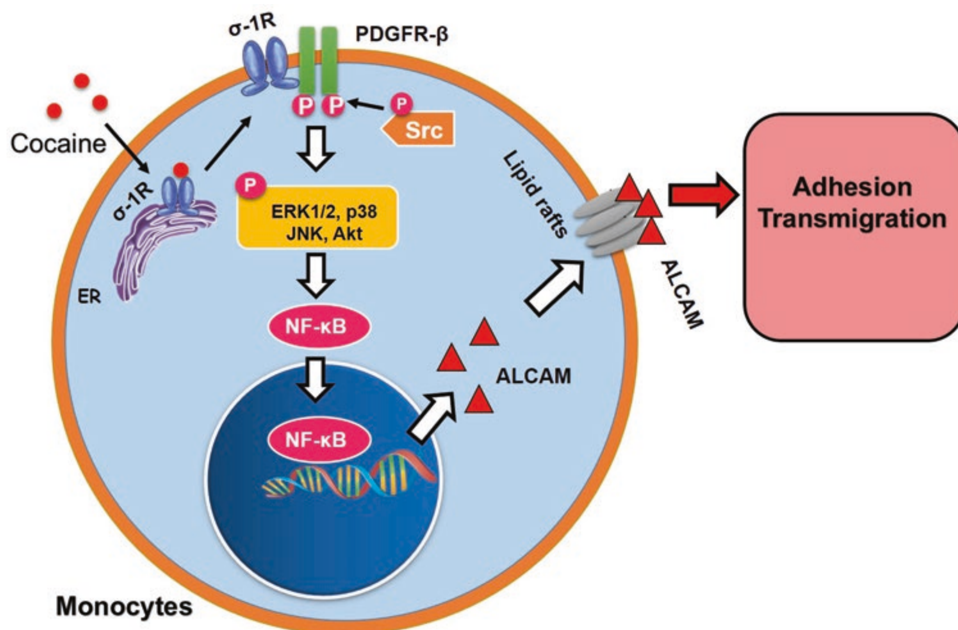

Fig. 12.2 Schematic of the signaling pathways involved in cocaine-mediated induction of ALCAM. Cocaine mediated induction of ALCAM involved translocation of Sig-1R to the plasma membrane, subsequent phosphorylation of PDGF-β (PDGF-β) followed by downstream activation of MAPK, Akt and NF-κB pathways

study from our lab has also found that translocation of the Sig-1R to the lipid raft micro-domains of the plasma membrane regulates cocaine-mediated induction of chemokine monocyte chemotactic protein-1 (MCP-1/CCL2) in microglia [19]. Taking advantage of pharmacological approach, we demonstrated that cocaine-mediated upregulation of MCP-1 expression resulted from activation of Src, mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3′ kinase (PI3K)/Akt and nuclear factor kappa B (NF-κB) pathways in a sequential manner.

ER stress is aberrant aggregation of misfolded proteins [32], and prolonged ER stress often results in neuro-inflammation [33]. Autophagy is a cellular adaptive mechanism in response to ER stress that delivers misfolded proteins and damaged organelles to the lysosome for degradation [34–39]. Accumulating evidence suggests that both ER stress and autophagy regulate neuroinflammation in various neurodegenerative disorders [40]. Interestingly, our recent study lends further credence to the fact that both ER stress and autophagy also play critical roles in cocaine-mediated release of inflammatory mediators in microglia [33]. Upregulation of autophagy-signature mediators such as Beclin1, ATG5, LC3-II was observed in both primary rat microglial cells *in vitro* and cocaine-injected mice *in vivo*. Moreover, both antagonists for autophagy, 3-methyladenine (3-MA) and Wortmannin, successfully blocked the release of inflammatory factors in microglial cells exposed to cocaine. We also observed attenuation of cocaine-mediated autophagy in microglial cells pretreated with ER stress inhibitor salubrinal, suggesting that ER stress was upstream of autophagy induced by cocaine. Taken together, these results suggested the involvement of ER stress-autophagy axis in cocaine-mediated neuroinflammation. Targeting at both Sig-1R and ER stress-autophagy axis thus has the therapeutic potential for treating neuroinflammation in HIV-positive cocaine users.

12.2.3 Astrocytes

Astrocytes are the most abundant cell type within the brain that play various active roles in the brain, including providing structural support for BBB, maintaining the homeostasis of neurotransmitters and ions and modulating synaptic transmission [41]. Toxic stimuli and traumatic insults in the CNS lead to proliferation of astrocytes and astrocytic hypertrophy with upregulation of filament protein glial fibrillary acidic protein (GFAP), known as "astrogliosis" [42]. Activated astrocytes release inflammatory factors, resulting in exacerbating neuroinflammation [43]. Accumulating evidence suggests that excessive intake of cocaine contributes to modulating the trajectory of HAND through astrogliosis [20, 44, 45]. We have recently succeeded in demonstrating that Sig-1R play an essential role in cocaine-mediated astrogliosis in HAND [20]. Immunostaining for GFAP in human postmortem cortex showed increased GFAP positive cells in HIV-positive cocaine users, compared with HIV-positive group without cocaine use. Both astrocytic cell line A172 and primary astrocyte culture recapitulates cocaine-mediated astrogliosis *in vitro*. These findings were corroborated by demonstrating upregulated GFAP in the cortex of cocaine-treated mice compared with saline injected controls. Furthermore, taking advantage of pharmacological approach, we showed that cocaine induces swift translocation of Sig-1R to plasma membrane, followed by mitogen-activated protein kinase (MAPK) signaling with subsequent downstream activation of the early growth response gene 1 (Egr-1). Activation of Egr-1, in turn, provokes transcription of GFAP. A better understanding of the cocaine/Sig-1R in mediating the astrogliosis is thus critical in dissecting the mechanism(s) underlying the disease progression of HAND and for future development of therapeutic targets.

12.2.4 Neurons

Despite the fact that direct infection of neurons by HIV-1 remains inconclusive, it has been well-known that one of the salient pathological characteristics for HAND is neuronal degeneration induced by viral proteins and virus-associated inflammatory conditions [46]. Accumulating evidence implicates that cocaine abuse potentiates neurotoxicity in the presence of HIV-1 viral proteins such as gp120 [21, 47]. Previous studies from our group have demonstrated the cocaine and gp120-mediated synergistic cellular toxicity on rat primary neurons [21]. Mechanistic study has also revealed the involvement of reactive oxygen species and loss of mitochondrial membrane in the combinatorial neurotoxicity induced by cocaine and gp120. Interestingly, mitogen-activated protein kinases (MAPK) signal pathways also plays an essential role in this process. Using pharmacological inhibitors, our group has further found that c-jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK)/MAPK pathways converge in the activation of nuclear factor (NF)-kB, leading to neuronal death. Another study from our group also reported that cocaine collaborated with gp120 boosts dendritic beading in rat primary hippocampal neurons, ultimately culminating into the formation of dendritic varicosity [47]. In summary, cocaine potentiates neurotoxicity mediated by HIV viral proteins and determining the detailed molecular mechanism(s) will provide insights for the development of new therapeutic approaches aimed at treatment of HAND in the drug-abusing population.

12.3 Sigma-1 Receptors in Other Neurodegenerative Disorders

12.3.1 Alzheimer Disease (AD)

AD, clinically characterized as an ongoing cognitive impairment, is the most common neurodegenerative diseases globally, with 46.8 million people affected worldwide [6]. Accumulation of neurofibrillary tangles containing hyperphosphorylated tau and Aβ plaques are two cardinal pathological features of AD [48]. Both postmortem and *in vivo* brain imaging studies have dem-

onstrated reduced density of Sig-1R in the brains of patients with AD [49, 50]. Interestingly, Sig-1R density remains unchanged during physiological aging, suggesting a possible correlation between reduction of Sig-1R and the pathogenesis of AD [51]. The etiology of Sig-1R loss however, remains unclear. It is known that the E4 variant of the apolipoprotein E gene (APOE 4) is a commonly recognized genetic risk factor accountable for certain fraction of late-onset AD [52], but it remains inconclusive whether this variant is attributable to a low density of Sig-1R in AD. Interestingly, in both Australian and Chinese cohorts it has been demonstrated that the interaction of Sig-1R and APOE 4 influences AD severity [53]. In contrast, studies on cohorts of Polish and Hungarian AD patients did not show significant evidence in support of interaction between Sig-1R and APOE 4 in AD [54, 55]. Further studies are required to address whether ethnicity/genetic diversity should be considered as a crucial contributing factor for the interaction between Sig-1R and APOE 4 polymorphism in the pathogenesis of AD.

Taking advantage of different Sig-1R agonists, accumulating evidence suggests a neuroprotective role of Sig-1R in AD through various mechanisms, including regulation of intracellular calcium, prevention of oxidative stress and anti-apoptotic effects. For example, pan selective Sig-1R agonist afobazole inhibits the increase of intracellular calcium level, suppresses nitric oxide (NO) production and lowers expression of the proapoptotic protein Bax and caspase-3 in rat cortical neurons exposed to amyloid beta_{25-35} ($A\beta_{25-35}$) [56]. Interestingly, afobazole also plays an anti-inflammatory role by decreasing microglial activation and migration and preventing apoptosis induced by Aβ25–35 in rat microglia [57]. ANAVEX2-73, a mixed muscarinic and Sig-1R agonist, was also reported to block Tau hyperphosphorylation and $A\beta_{1-42}$ production in $A\beta_{25-35}$-injected mice [58]. In the same mouse model of AD, ANAVEX2-73 has also been shown to prevent oxidative stress and learning deficits [59]. However, knocking down of Sig-1R affects survival of primary hippocampal neurons and leads to degeneration [60], further suggesting a neuroprotective role of Sig-1R. Clinically approved AD drug donepezil has also been demonstrated to protect memory function synergistically with Sig-1R agonists PRE-084 or ANAVEX2-73 in mice treated with $A\beta_{25-35}$ [61]. A better understanding of the role of Sig-1R in AD is thus critical in dissecting the mechanism(s) underlying disease pathogenesis and for future development of therapeutic targets.

12.3.2 Parkinson Disease (PD)

PD is well recognized as the second most common neurodegenerative disorder characterized by bradykinesia, rigidity and resting tremors. The majority of the symptomatology of the disease are attributable to the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in the impairment of dopaminergic neurotransmission [62, 63]. Similar to the findings in AD, reduced Sig-1R density and dopamine release has also been observed in early PD patients using [^{11}C] SA4503 and positron emission tomography (PET) [64]. Interestingly, a link between Sig-1R and dopamine has been elegantly reported by Mori *et al.* in his study, which showed that dopamine at physiological concentration (lower than 10 μM) induces apoptosis in Chinese hamster ovary (CHO) cells that were knocked down for the sigma 1-R, but not in the wildtype CHO cells [65]. The possible mechanism underlying apoptosis involved dopamine mediated conversion of nuclear factor κB (NF-κB) p105 to the active form of p50 in the proteasome of Sig-1R knockdown CHO cells, leading in turn, to downregulation of Bcl-2 and apoptosis. Loss of Sig-1R could thus render neuronal cells vulnerable to drug-induced dopamine surge or even physiological dopamine level, resulting in turn, to dopamine toxicity [65]. Based on these data, it can be envisioned that Sig-1R and its ligands could be developed as potential therapeutic targets for PD. PRE-084 is a selective Sig-1R agonist and has been demonstrated to restore behavioral performance as well as neuronal function in PD mouse models with intrastriatal 6-hydroxydopamine (6-OHDA) lesions [66].

PRE-084 upregulates neurotrophic factors (Brain-derived neurotrophic factor, BDNF and glial cell line-derived neurotropic factor GDNF) and their downstream, and modestly recovers dopamine levels, followed by increased density of dopaminergic fibers in striatal regions.

Levodopa is a commonly used drug for long-term treatment of PD but is often accompanied by dyskinesia, which is known as levodopa-induced dyskinesia (LID). A PET study has also revealed abnormally elevated binding potentials of cerebellar sigma receptors in advanced PD patients, suggesting involvement of sigma receptors in the pathogenesis of LID [67]. It is not surprising due to the fact that Mori *et al.* has also demonstrated that dopamine can significantly upregulate Sig-1R expression and ER chaperone protein in CHO cells in a dose-dependent manner [65]. Sig-1R antagonist BMY-14802, previously used for treating schizophrenia, has been demonstrated to reduce abnormal involuntary movement and improve motor functions in the 6-OHDA rat model of PD through serotonin 5-HT$_{1A}$ receptor [68].

Sig-1R plays an important role in maintaining the balance of the dopaminergic system in the brain. Restoring the homeostasis of Sig-1R might provide insights for developing potential therapeutic targets for PD or LID. However, the intervention in the Sig-1R signaling pathway through various Sig-1R ligands should be carefully investigated because some ligands such as trishomocubanes and safinamide do not necessarily exert neuroprotective effects or improve behavioral performance through Sig-1R [69, 70].

12.3.3 Amyotrophic Lateral Sclerosis (ALS)

ALS is a progressive neurodegenerative disorder characterized by loss of spinal cord motor neurons (MNs), leading to weakness in the muscles and eventually death from respiratory failure [71, 72]. It is often accompanied with other neurodegenerative diseases such as Frontotemporal Lobar Dementia (FTLD) [73]. Annually, the prevalence of ALS in United States is 3.9 cases per 100,000 general population, and it is more prevalent among persons aged 60-69 years [8]. Although major advances have been made in our understanding of the genetic causes of ALS, the pathophysiology of this disease still remains poorly understood. A number of genes have been identified and associated with the establishment of ALS including superoxide dismutase-1 protein (SOD1), RNA-binding protein Fused in Sarcoma (FUS), TAR DNA-binding protein 43 (TARDBP) and [74, 75]. Under normal conditions, Sig-1R is particularly enriched in MNs present in the brain stem and spinal cord [76, 77], while mutations in this gene have been found to contribute to the pathogenesis of FTLD-ALS and juvenile ALS [78, 79]. Prause *et al*. examined the expression of Sig-1R in *post mortem* spinal cord of ALS patients and in the SOD1 transgenic mouse model of ALS, and found presence of abnormally accumulated Sig-1R in enlarged C-terminals and endoplasmic reticulum structures of alpha MNs, which further supported the association of abnormally modified Sig-1R with ALS [80].

Information gleaned from studies in ALS patients and from various ALS models in the past years, has shed light on the role of mitochondrial damage and oxidative stress, excitotoxicity, neuroinflammation, ER stress, misfolded protein aggregation and defective removal of toxic proteins as the pathological hallmarks of ALS [81], Most of these processes have been shown to be modulated by Sig-1R. For example, Mavlyutov *et al*. demonstrated that Sig-1R acts as a brake on MN excitability in the SOD1 G93A mouse model of ALS, while the reduced excitability may extend the longevity of MN. This is the first case wherein the absence of Sig-1R has been shown to be attributable to the shortened lifespan of ALS in the mouse model [82]. Intriguingly, Prause et al. have also indicated that disturbances in the unfolded protein response and impaired protein degradation were related to the accumulation of Sig-1R in cultured human ALS-8 skin fibroblasts and SOD1 transgenic mouse alpha motor neurons. Furthermore in this study, deranged calcium signaling and abnormalities in ER and Golgi structures caused by shRNA knockdown of Sig-1R have also been reported to result in the apoptosis of motor neurons [80].

Interestingly, in several published reports, Sig-1R agonist PRE084 has been implicated as a potential therapeutic strategy for neuroprotection in the ALS mouse model. For example, Mancuso *et al.* demonstrated that administration of PRE084 (0.25 mg/kg, i.p.) improved functioning of motor neurons and extended their survival in both female and male SOD1-G93A ALS mice [83]. In another study a similar therapeutic effect of PRE084 administration in SOD1-G93A ALS mice was reported and it was also shown that Sig-1R mediated neuroprotective effects on the motor neurons by reducing the number of activated astrocytes and macrophage/microglia [84].

12.3.4 Huntington's Disease (HD)

HD, a devastating, hereditary neurodegenerative disease, affects approximately five to seven out of every 100,000 people in the Western countries [9]. As HD is a hereditary disease, children have a 50 % chance of inheriting the genetic trait from an affected parent. HD is caused by over expansion of a cytosine-adenine-guanine (CAG) trinucleotide repeat in the huntingtin gene, which is normally less than 27 repeats. CAG repeats expand through replication error to 40 or more are fully penetrant and inevitably associated with neuronal degeneration and the progressive motor, cognitive, and behavioral features of HD [85].

Normally, huntingtin shuttles between the ER and the nucleus, and plays a role in regulation of autophagy triggered by ER stress. However, mutated huntingtin loses its ability to return to the ER and starts to aggregate in the nucleus [86–88]. A recent study demonstrated that accumulation of Sig-1R is a feature common for mediating neuronal nuclear inclusions in HD, which thereby implicated Sig-1R in the ER-related degradation machinery for the mutated huntingtin [89]. Additionally, Hyrskyluoto *et al.* reported that expression of mutant huntingtin resulted in decreased Sig-1R levels in a neuronal cell line (PC6.3), which in turn, could be restored by the administration of Sig-1R agonist PRE084. These findings suggested that Sig-1R agonist PRE084 elicits beneficial effects in models of HD via positively affecting NF-kB signaling to upregulate the levels of cellular antioxidants and by decreasing ROS levels [90]. Furthermore, it is well known, that the dopamine stabilizer - ACR16 is in an advanced phase of clinical trials for the relief of the motor symptoms of Huntington's disease [91]. This drug is thought to exert its beneficial effects primarily via the dopamine D2 receptor. Interestingly, Sahlholm *et al.* have provided a novel idea that ACR16 binds Sig-1R in low concentrations, which is 100 times lower than that reported for its interaction with the D2 receptor [92]. This new knowledge could be used to develop future treatments for HD. Overall, Sig-1R could be envisioned as a promising target for future drug development for HD.

12.4 Conclusions

Both *in vitro* and *in vivo* studies have significantly advanced our understanding of the molecular mechanism(s) underlying Sig-1R and have revealed the important role of Sig-1R in both neurodegenerative disorders as well as in cocaine abuse. Restoring the homeostasis of Sig-1R could provide insights for developing potential therapeutic targets for neurodegeneration and for cocaine-related neurologic impairments. It must be cautioned however, that the molecular regulation of Sig-1R pathways still remains to be elucidated in detail. The intervention in the Sig-1R signaling pathway by various Sig-1R ligands should be carefully investigated because some ligands with high affinity do not necessarily exert neuroprotective effects or improve behavioral performance through Sig-1R [69, 70].

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13 Sigma Receptors and Substance Use Disorders

Valentina Sabino, Callum Hicks, and Pietro Cottone

Abstract

Thanks to advances in neuroscience, addiction is now recognized as a chronic brain disease with genetic, developmental, and cultural components. Drugs of abuse, including alcohol, are able to produce significant neuroplastic changes responsible for the profound disturbances shown by drug addicted individuals. The current lack of efficacious pharmacological treatments for substance use disorders has encouraged the search for novel and more effective pharmacotherapies. Growing evidence strongly suggests that Sigma Receptors are involved in the addictive and neurotoxic properties of abused drugs, including cocaine, methamphetamine, and alcohol. The present chapter will review the current scientific knowledge on the role of the Sigma Receptor system in the effects of drugs and alcohol, and proposes that this receptor system may represent a novel therapeutic target for the treatment of substance use disorders and associated neurotoxicity.

Keywords

Cocaine • Methamphetamine • Alcohol OR Ethanol • Alcoholism • Addiction • Withdrawal • Drug abuse OR Abused drug • Psychostimulant

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13.1 Introduction

The abuse of licit and illicit substances has a profound impact on the health of people worldwide. The United Nations Office on Drugs and Crime (UNODC) indicates that about 230 million people, or 5 % of the world's adult population, are estimated to have used an illicit drug at least once in 2010 [1]. The same report states that ~1 % of all global deaths among adults is attributed to illicit drug use [1]. The global status report on alcohol and health by the World Health Organization (WHO) indicates that worldwide alcohol consumption in 2010 was equal to 6.2 l of pure alcohol consumed per person aged 15 years or older per day [2]. WHO also reports that in 2012, over 3 million deaths (~6 % of all global deaths) were attributable to the consumption of alcohol [2].

Substance use disorder, as defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), is a cluster of cognitive, behavioral, and physiological symptoms indicating that the individual continues using the substance despite significant substance-related problems [3]. In the DSM-5, each specific substance is described individually (e.g., alcohol use disorder, stimulant use disorder), but nearly all substances are diagnosed based on the same overarching criteria. Indeed, the manual lists eleven criteria for the diagnosis of each disorder, which are clustered within four different groups: impaired control, social impairment, risky use, and pharmacological criteria [3].

Thanks to advances in neuroscience, addiction is now recognized as a chronic brain disease with strong genetic, neurodevelopmental, and sociocultural components [4]. Drugs of abuse, including alcohol, are thought to exert their initial reinforcing effects by causing sharp increases in dopamine in reward-related brain regions, thus "hijacking" the reward substrates originally evolved to subserve natural rewards [5]. Repeated drug consumption produces neuroplastic alterations which underlie the profound disruptions observed in drug addicted individuals, which include increased reactivity to drug cues, reduced sensitivity to non-drug rewards, weakened self-control, and increased sensitivity to stressful stimuli.

Therapeutic interventions intended to alleviate the above described impairments would, therefore, be beneficial for the treatment of addiction [6]. However, the limited pharmacological treatments currently available for substance use disorders suffer from limitations related to poor efficacy and/or adverse side effects [7]. This has encouraged the search for more effective pharmacotherapies for drug addiction that interact with novel molecular targets.

Sigma receptors (SigRs) have been implicated in both the addictive and the neurotoxic properties of abused drugs. Although the majority of studies have focused on cocaine and methamphetamine, growing evidence has highlighted a role for this receptor system in the effects of other drugs such as hallucinogens and ethanol. Therefore, SigRs and associated ligands are being investigated as potential therapeutic targets for substance use disorders. This chapter will review the major findings in the current literature in relation to the interaction between SigRs and illicit drugs of abuse as well as alcohol. Two subtypes of SigR have been described to date, Sig-1R and Sig-2R. The functional relevance of the Sig-1R subtype in specific actions of drugs of abuse has been supported by knock down studies using either oligonucleotide or siRNA approaches. Since only the Sig-1R has been cloned so far, and because of the limited availability of ligands selective for the Sig-2R, much less is known about the role of the latter subtype in the effects of abused drugs; therefore, this chapter will focus mainly on the Sig-1R subtype. In addition, it is important to note that compounds labeled here as "Sig-1R" or "Sig-2R" because of their preferential affinity for one or the other subtype, when given *in vivo*, likely reach concentrations sufficient to activate/inhibit both subtypes.

13.2 Sigma Receptors and Cocaine

Cocaine is an alkaloid derived from the coca plant (*Erythroxylum coca*) with very powerful psychostimulant effects [8]. Cocaine has strong rewarding and reinforcing properties and as such,

is one of the most abused illegal drugs. Estimates by the United Nations Office on Drugs and Crime (UNODC) in 2011 indicate that 17 million people used cocaine at least once in the past year within North America, Western, and Central Europe, together accounting for approximately one half of cocaine users worldwide [9]. Cocaine users show a variety of psychiatric conditions including addiction, depression, and anxiety disorders, as well as negative psychosocial and physical consequences [8]. The most important harmful physical consequences associated with cocaine use include convulsions and fatal overdose [8, 10–12].

13.3 Molecular Mechanism

At a molecular level, cocaine is known to interact with several target proteins. The most relevant mechanism of action of cocaine is the inhibition of serotonin, norepinephrine, and dopamine reuptake at synapses, which underlies the sympathomimetic, locomotor stimulating, and rewarding/reinforcing properties of the drug. Relevant to the topic of this chapter is a large body of evidence that demonstrates molecular interactions between Sig-1Rs and cocaine. The most prominent evidence is a direct binding of cocaine to SigRs, which was first described in 1988 [13] and then later confirmed by several other reports [14–17]. Due to the reported low (i.e. micromolar) affinity of cocaine for SigRs, the physiological relevance of this binding was initially controversial. However, not only is it now well-established that such concentrations of cocaine are achieved in the body [18, 19], but recent studies also suggest that the Sig-1R occupancy by cocaine *in vivo* may be higher than that suggested by *in vitro* studies. In particular, according to a recent study, the *in vivo* ED50 of cocaine for the Sig-1R is only 2.6-fold lower than that for the dopamine transporter (DAT) in the mouse brain [14]. Following binding to the Sig-1R, cocaine displays an agonistic profile and SigR antagonists can block its effects. Interestingly, cocaine has been shown to bind SigRs that form complexes with other proteins. Specifically, cocaine binds Sig-1R/dopamine D2R heteromers inhibiting the ERK1/2 intracellular signaling [20], but also binds Sig-1R/dopamine D1R/histamine H3 receptors complexes, disinhibiting the H3 receptor-mediated brake on D1R signaling [20]. In addition, cocaine triggers the formation of the heteromer Sig-1R/Kv1.2 channel, a phenomenon associated with the redistribution of both proteins from intracellular compartments to the plasma membrane [21].

Cocaine-induced upregulation of the immediate-early gene fos-related antigen 2 (fra-2) can be prevented by treatment with the Sig-1R antagonist BD-1063 [22]. In addition, following cocaine stimulation, the Sig-1R translocates from the endoplasmic reticulum to the nuclear envelope, where it binds the protein emerin to form a molecular complex through the recruitment of the gene repressor specific protein 3 (Sp3), chromatin-remodeling factors (lamin A/C, barrier-to-autointegration factor (BAF), and histone deacetylase (HDAC)), thereby modulating cocaine-induced transcriptional regulation [23]. Altogether, these studies indicate that the Sig-1R has a role in the transcriptional regulation through which cocaine may exert its addictive actions.

13.4 Toxic Effects

A large body of evidence shows that SigRs mediate the toxic, locomotor-stimulating, rewarding, and reinforcing effects of cocaine, demonstrating the potential of SigR antagonists as therapeutic agents for cocaine-related pathologies [24–26].

In the context of SigRs, convulsions and lethality represent the most widely studied cocaine-induced toxic effects. Cocaine-induced seizures occur in 1–8 % of all cocaine users with or without a history of epilepsy, and they are not necessarily associated with cocaine overdose [10–12]. Data from the National Center for Health Statistics at the Centers for Disease Control and Prevention indicate that in 2014, more than 5000 overdose deaths involving cocaine have been recorded in the USA, and that a 42 % increase in the total number of deaths from 2001 to 2014 was observed [27].

SigR antagonists have been shown to exert protective effects against cocaine-induced toxicity, as they are able to significantly reduce cocaine-induced convulsions and lethality. Furthermore, the knock down of Sig-1Rs with antisense oligodeoxynucleotides attenuates the pro-convulsive effects of cocaine [28–33].

Interestingly, a bidirectional relationship between cocaine-induced toxicity and SigRs exists, as convulsions and lethality are worsened by SigR agonists at doses that are *per se* inert. In particular, pretreatment with the SigR agonists DTG, BD-1031, and BD-1052 was shown to exacerbate cocaine-induced toxic effects [28, 29].

13.5 Locomotor Effects

Another widely studied effect induced by cocaine is locomotor activation that represents a direct measure of the psychostimulant properties of the drug. Cocaine-induced locomotor activity is mediated by mesolimbic structures including the striatum, and represents an important behavioral output related to the abuse liability of the drug. The locomotor stimulating effects of cocaine can be studied in experimental protocols using either drug-naïve individuals or subjects who have already been exposed sub-chronically or chronically to cocaine. The latter procedure is known as locomotor (or behavioral) sensitization, as individuals pre-exposed to cocaine "sensitize", exhibiting an enhanced locomotor response once re-exposed to cocaine. This protocol is particularly useful to study the cellular and molecular neuroadaptive mechanisms induced by repeated cocaine use.

Numerous studies have demonstrated the involvement of SigRs in the locomotor stimulant effects induced by cocaine. In particular, several Sig-1R antagonists have been shown to block the acute locomotor-stimulating effects of cocaine [15, 28, 30, 32]. These effects typically occur at doses that have no intrinsic locomotor actions, suggesting that SigRs modulate the psychostimulant effects of cocaine, rather than mediating this physiological behavioral response. The effects obtained by pharmacologically blocking SigRs can also be achieved by knocking down the receptor through the use of antisense oligodeoxynucleotides [28].

SigR antagonists have also proven effective in counteracting the locomotor sensitizing effects of cocaine [21, 30, 34]. It was recently shown that blockade of the Sig-1R site specifically within the nucleus accumbens (NAcc) prevents the locomotor sensitizing effects of cocaine [35]. Interestingly, following repeated administration of cocaine in a locomotor sensitization procedure, gene and protein expression of the Sig-1R increase in both the striatum and the cortex [34], and results in the formation of a Sig-1R/Kv1.2 channel heteromer [21].

While SigR antagonists do not affect locomotor activity *per se*, some agonists have been shown to exert locomotor-stimulating effects [36, 37]; this observation is consistent with the hypothesis that the stimulating effects of cocaine may also be mediated by its agonistic binding to the Sig-1R. In addition, similar to cocaine, Sig-1R agonists have been shown to enhance dopamine release in the striatum [37], an effect that is thought to mediate the locomotor-activating effects of psychostimulants.

13.6 Rewarding Effects

Cocaine possesses strong rewarding properties, and also has the ability to increase the salience of the contextual stimuli associated with the drug through a Pavlovian associative learning process. Through this mechanism, contextual neutral stimuli that can include paraphernalia, places, or people, acquire rewarding properties and exert a strong control over behavior even in the absence of cocaine. This process is thought to play a critical role in maintaining drug-taking behavior, as the approach to a drug-associated context sets the occasion for drug-taking behavior to be engendered [38]. A widely used experimental procedure to evaluate the rewarding properties of cocaine is the conditioned place preference (CPP) paradigm, in which an environment equipped with specific contextual cues, following repeated pairings with the drug, becomes preferred as compared to another neutral environment in the absence of the

drug [38]. Cocaine is able to induce a robust CPP, measured as the time spent in the drug-associated environment as compared to the time spent in the neutral environment. To test the ability of pharmacological agents to block the rewarding properties of cocaine, two experimental procedures can be used: either the pharmacological agent can be administered before each pairing to block the acquisition of conditioning, or it can be given before the expression of the conditioned preference. Sig-1R antagonists including NE-100, BD-1047, AC927, and CM156, have been shown to successfully block both the acquisition and the expression of cocaine place preference, without affecting place conditioning *per se* [30, 39–43]. Blockade of cocaine-induced CPP is also obtained using an antisense probe targeting the Sig-1R or the neurosteroid progesterone, which acts as an antagonist at the Sig-1R [44]. Interestingly, antagonism of the Sig-1R enhances the ability of social interaction to inhibit the place preference produced by cocaine [45]. It has also been shown that pretreatment with the Sig-1R antagonist BD-1047, or repeated treatment with an antisense probe targeting the Sig-1R, prevents the reactivation of CPP induced by cocaine priming. In this procedure, cocaine-induced CPP is initially extinguished through repeated exposure to the drug-paired context in the absence of the drug, and then is reactivated by priming subjects with cocaine. Using the same procedure, cocaine-induced CPP was reactivated by the Sig-1R agonist igmesine or the steroid dehydroepiandrosterone (DHEA), and blocked by pretreatment with the Sig-1R antagonist BD-1047 [43]. Interestingly, repeated cocaine treatment in the CPP procedure increased Sig-1R gene expression in the nucleus accumbens [40]. Also, antagonism of the Sig-1R blocks the alterations in whole brain gene expression observed following cocaine-induced place preference as measured using a microarray gene profiling [42]. Altogether, these reports suggest that the Sig-1R is able to modulate the rewarding properties of cocaine.

In contrast with their effects on locomotor activity, agonists of SigRs do not produce CPP *per se* [40]. Interestingly, a recent report has shown that the "atypical" Sig-1R agonist SA4503, but not (+)-pentazocine, is able to attenuate the acquisition of cocaine-induced CPP [46].

13.7 Reinforcing Effects

Cocaine is not only a strong reward but also a strong reinforcer (a substance whose effects increase the likelihood that use will reoccur). The reinforcing effects of drugs are studied in instrumental conditioning, another form of associative learning that is significantly different from Pavlovian conditioning. In instrumental conditioning, subjects learn to "self-administer" a substance by working on a manipulandum, typically a lever, within a Skinner box (also known as an operant chamber). By varying the paradigms and the schedules of reinforcement, experimenters are able to measure not only whether a substance is reinforcing, but also the strength of reinforcement, whether cues associated with the drug can reinstate responding after its extinction, and whether drugs share similar subjective effects.

In drug discrimination studies that aim to assess the similarity of the subjective effects of specific drugs, SigR agonists have failed to produce full cocaine-like discriminative-stimulus effects. For example, SA-4503, PRE-084, and DTG all failed to substitute for the cocaine discriminative stimulus [47–49], suggesting that differences in neurochemical effects of cocaine and SigR agonists may contribute to their different subjective effects. Notably, DTG was shown to shift the cocaine substitution curve to the left, suggesting that SigR activation produces an augmentation of the discriminative stimulus properties of cocaine [48].

Contrary to what was observed with the locomotor-stimulating or the rewarding effects of cocaine, antagonists of Sig-1Rs including BD-1047, BD-1063, NE-100, and AC 927, do not seem to be effective in reducing the reinforcing efficacy of cocaine [50–52]. However, this discrepancy is not necessarily contradictory. Indeed, each of these behavioral endpoints is substantially different from the others, and each represents only one drug-related behavioral output. This highlights the fundamental differences in the mechanisms underlying the rewarding and

reinforcing effects of cocaine [26]. In addition, while most of the studies showing effects of SigR ligands were performed in mice, self-administration studies used rats.

While SigR antagonists exert no effect on cocaine self-administration, SigR agonists such as DTG and PRE-084 are able to shift the cocaine dose-response curve to the left, indicating that they increase the reinforcing efficacy and therefore the potency of cocaine [51]. Interestingly, SigR agonists are not self-administered by rats, unless the subjects already have a history of cocaine self-administration [51, 53]. Once established, SigR antagonists block the reinforcing effects of SigR agonists. These effects are independent of dopamine, as dopamine antagonists do not affect self-administration of the SigR agonist PRE-084. Interestingly, in both cocaine-naïve and cocaine experienced subjects, self-administration of SigR agonists does not appear to be accompanied by release of dopamine in the NAcc, except when high doses of PRE-084 are used [53, 54]. The results discussed above indicate that experience with cocaine is able to induce reinforcing effects of previously inactive SigR agonists, and therefore the actions of cocaine on SigRs may contribute to its addictive properties [26].

Notably, the Sig-1R antagonist BD-1047 is able to block cocaine-seeking behavior in rats in a cue-induced reinstatement procedure, in which responding for cocaine is first extinguished and then reinstated through the use of cues specifically associated with the drug [50]. This suggests that the ability of cues to reinstate cocaine-seeking behavior involves the activation of SigRs, and suggests that SigR antagonists may represent a therapy to prevent relapse to cocaine.

13.8 Sigma Receptors and Methamphetamine

Amphetamine type stimulants (ATS) have become the focus of increasing attention. The use of ATS, in particular methamphetamine (MA) and “ecstasy”, are widespread and rising globally, making ATS the second most widely used class of illicit drugs worldwide [9]. MA is a potent stimulant with high abuse potential. MA is among the most popular psychostimulants in the world, and its abuse has reached epidemic proportions [9, 55]. MA use is associated with increased risk of early mortality, increased risk of heart disease, and greater likelihood of engaging in high-risk sexual behaviors [56–60]. Chronic MA abuse is associated with serious medical conditions affecting multiple organ systems [61]. MA-related complications involve cardiovascular risks (chest pain, arrhythmias, hypertension, cardiomyopathy, and acute myocardial infarction, accelerated coronary artery disease, cardiac hypertrophy), neurological symptoms (seizures, hyperkinesia, stereotyped behaviors), dental issues, and dermatological issues [57, 62–66].

MA use is also associated with global neuropsychological and cognitive impairment, which include deficits in executive functions, memory, attention, language, and psychomotor function [67–70]. Chronic MA abuse is also associated with high rates of comorbid psychiatric symptoms [71, 72], psychotic symptoms, mood and anxiety disorders, suicide attempts, as well as ADHD [73–79]. As a central nervous system stimulant, MA causes euphoria, increased energy and alertness, and decreased appetite [80].

13.9 Molecular Mechanism

The most widely accepted molecular mechanism of action of MA is related to its ability to increase the release of monoamines such as serotonin, norepinephrine, and dopamine at synapses, through actions on the plasmatic reuptake transporters. MA binds monoamine transporters and its reuptake inside the cell alters the function of the vesicular monoamine transporter 2, which culminates in the release of monoamines from the vesicles to the cytoplasm. The increase in monoamines in the cytoplasm reverses the directionality of transporters, causing neurotransmitter release [81–84]. In addition to this mechanism, cell-based binding assays have demonstrated that MA directly interacts with SigRs. Moreover, *in vitro* competition binding assays have shown that MA interacts with SigRs at relevant (micromo-

lar) concentrations, but exhibits a slight preference for the Sig-1R (2 μM) compared to the Sig-2R (47 μM) [85], and appears to act as a competitive agonist. MA may also interact with SigRs indirectly, either by directly regulating other endoplasmic reticulum proteins (e.g. BiP) or by regulating D1 receptor-mediated processes, which in turn have been shown to physically interact with SigRs [86–88].

The interaction between MA and the Sig-1R has also been reported in terms of molecular adaptations following prolonged MA use. An early study [89] found that repeated (10-day) MA administration caused a significant up-regulation of Sig-1R in several brain regions of rats including the substantia nigra (SN), the frontal cortex, and the cerebellum. A later study showed that rats who had self-administered MA for 5 weeks had significantly increased levels of Sig-1R protein in the midbrain, as well as decreased and increased Sig-1R mRNA in the frontal cortex and hippocampus, respectively [90]. On the other hand, rats that received experimenter-delivered injections of MA or saline showed no alterations in Sig-1Rs. However, Hayashi and colleagues [91] subsequently found that passive injections of MA in one group of rats each time an infusion was actively self-administered by another group of rats ("yoked" self-administration) was sufficient to up-regulate Sig-1Rs in the ventral tegmental area (VTA) and the SN.

13.10 Toxic Effects

The leading cause of death following MA overdose is hyperthermia [92], an effect that is also associated with increased neurotoxicity [93, 95]. MA dose-dependently increases body temperature in laboratory animals, and this effect can result in death [92].

Preclinical studies have demonstrated that the hyperthermic effect of MA is partly mediated by SigRs. The SigR agonist DTG has been shown to increase body temperature but interestingly, despite increasing the lethal effects of MA, does not appear to worsen MA hyperthermia [93]. Several SigR antagonists including AC927, SN79, CM-156 and AZ-66 have all been shown to attenuate MA hyperthermia [93–99] and at higher doses, can also reduce body temperature [93]. The cognitive impairment resulting from a neurotoxic dosing regimen with MA can also be prevented by the Sig-1R antagonist AZ-66 [96].

MA neurotoxicity is associated with its long-term damaging effects on dopaminergic and serotonergic axon terminals in the striatum, hippocampus, and prefrontal cortex [100–102]. In addition, there is supporting evidence that MA may produce cell death [103]. The mechanism by which the SigR is involved in the neurotoxic and hyperthermic effects of MA is not yet known. However, SigR antagonists have been shown to significantly attenuate MA-induced DA and 5-HT nerve terminal degeneration toxicity, as reflected by striatal DA and 5-HT depletion and reductions in the expression levels of their transporters [93, 94, 99, 104]. The neuroprotective action of SigR antagonists has also been proposed to involve activation of caspases, as well as a reduction in reactive oxygen species and reactive nitrogen species production [105].

MA causes microglial activation in DA innervated areas, triggering the release of several pro-inflammatory cytokines that can lead to glial dysfunction as well as neuronal death. MA has also been shown to induce astrogliosis in the striatum and hippocampus [106–108]. Previous research suggests these effects may partially involve activation of SigRs. Indeed, MA-induced stimulation of astrocytes *in vitro* was blocked by pretreatment with the Sig-1R antagonist BD-1047 [109]. In addition, MA failed to activate astrocytes in Sig-1R knockout (KO) mice *in vivo*, but triggered an immune response in WT mice [109]. Male rats and mice subjected to a neurotoxic regimen of MA also show increased astrogliosis [104, 110], which is attenuated by pretreatment with the Sig-2R antagonist SN-79 [104].

13.11 Locomotor Effects

Repeated MA use can result in behavioral abnormalities spanning the sensorimotor, perception, and social behavior domain. Unlike non-human

primate models that offer more behavioral repertoires sensitive to the effects of MA, most rodent studies have focused on motor activity and stereotypy. As the acute dose of MA increases, its behavioral effects in rodents generally shift from the induction of hyperactivity to the induction of stereotypy. These effects of MA become exaggerated with repeated treatment, a phenomenon termed sensitization [111–114].

The Sig-1R agonist pentazocine was shown to potentiate stereotyped behavior produced by MA [115]. However, this effect does not appear to be shared by the agonists PB 28 and (+)-SKF 10,047, which failed to significantly alter repetitive movements induced by MA [116]. The Sig-1R antagonists BMY-14802 and BD-1047 have been shown to alter the pattern of MA-induced stereotypy, producing a shift from stereotypical biting to stereotypical sniffing, without affecting the overall frequency of stereotypical behavior. On the other hand, the selective Sig-2R antagonist SM-21 had no effect on MA-induced stereotypy [116]. Importantly, the increase in the intensity of stereotypy following repeated MA administration was prevented by the Sig-1R antagonist BMY-14802 [117], suggesting that Sig-1Rs are involved in MA-induced sensitization. Interestingly, the Sig-1R agonist (+)-3-PPP was shown to induce more pronounced stereotyped movements in rats previously sensitized with MA than in saline-pretreated rats, suggesting that repeated MA treatment induces persistent hypersensitivity of Sig-1Rs [118].

Evidence from a number of pharmacological studies demonstrates that MA exerts its locomotor stimulatory effect, at least in part, via Sig-1Rs. Mice administered the Sig-1R antagonists BD-1063 and BD-1047, as well as an antisense oligonucleotide to down-regulate brain Sig-1Rs, exhibited a reduced locomotor stimulatory response to MA [85]. Consistent with the hypothesis that MA-induced locomotor stimulation involves the activation of Sig-1Rs, the Sig-1R agonist SA-4503 enhanced the stimulatory effects of MA when injected at a low dose. However, at higher doses SA-4503 was able to reduce MA-induced hyperactivity, although a trend towards a reduction in motor activity *per se* could be observed [119].

Repeated MA injections induce behavioral sensitization, which may be mediated by SigRs. Indeed, the Sig-1R antagonist BMY-14802 is able to prevent increases in stereotypy induced by MA. BMY-14802 also attenuated locomotor sensitivity to a MA prime after a 7-day abstinence period [117]. A similar result was found after administration of the selective Sig-1R antagonist MS-377, which was able to reduce the behavioral sensitization induced by repeated MA treatment in rats [120].

The molecular basis of action of SigR antagonists on psychostimulant-induced behaviors is uncertain, but it may involve the attenuation of dopaminergic transmission. SigR agonists have been shown to increase extracellular dopamine levels in the nigrostriatal system [36, 121, 122]. An exception to this is the Sig-1R agonist SA-4503, which was found to reduce MA-evoked dopamine release from striatal slices, consistent with its “atypical” inhibitory action on MA-induced behaviors [119]. Surprisingly, the Sig-1R antagonist BD-1063 and BD-1047 failed to alter both the spontaneous and the MA-evoked dopamine release from striatal slices [119]. These observations contradict previous reports showing that these antagonists produce inhibitory effects on dopamine systems in other brain areas such as the VTA and the hippocampus [123, 124]. Therefore, further research is needed to elucidate the mechanism by which SigR antagonists attenuate MA-induced locomotor effects.

13.12 Rewarding Effects

In drug discrimination studies where rats are trained to discriminate MA from saline, the Sig-1R agonist SA-4503 was shown to dose-dependently shift the stimulus-substitution curve for MA to the left by approximately 200-fold, suggesting that Sig-1R activation enhances MA discriminative properties [119]. Notably, SA-4503 did not alter the discriminative stimulus properties of d-amphetamine, suggesting that the

lower affinity of d-amphetamine for the Sig-1R compared to MA (Ki: >10 μM vs. 2.2 μM) may be responsible for the differences observed [85, 119, 125]. Interestingly, however, SA-4503 did not substitute for the MA discriminative stimulus [119]. These data, together with the previously discussed cocaine findings, confirm the notion of distinct subjective effects of SigR agonists to those of psychomotor stimulants.

Only one study has to date investigated the effects of SigR ligands on MA-induced CPP. Indeed, the Sig-1R agonist SA-4503 induces a paradoxical attenuation of MA-induced place preference in rats [46], such that when given alone, SA-4503 does not produce either place preference or place aversion. To date, SigR antagonists have not been tested on MA-induced place preference. Based on the observation that adaptations in SigR expression occur following MA self-administration, a possible involvement of SigRs in the reinforcing effects of MA is hypothesized [90]. However, the effects of SigR ligands on MA self-administration have not yet been reported.

13.13 Sigma Receptors and MDMA

The amphetamine derivative 3,4-methylenedioxy methamphetamine (also known as MDMA or “ecstasy”) has psychostimulant-like properties as well as a weak psychedelic effect, resulting from its actions on the dopamine and serotonin systems.

Unlike other psychostimulants such as cocaine and MA, only one study to date has examined the interaction between Sig-1Rs and MDMA. Using *in vitro* competition and saturation binding assays, it was shown that the binding affinity of MDMA for the Sig-1R and Sig-2R subtypes is in the micromolar range, and that it preferentially competes for binding at the Sig-1R, although it discriminates between the two SigR subtypes to a lesser degree compared to cocaine and MA [126]. The same authors also showed that in behavioral tests peripheral injection of the Sig-1R antagonist BD-1063 dose-dependently reduced the locomotor stimulant effects of MDMA in mice, shifting the MA dose–response curve to the right [126]. These findings suggest that MDMA may partly act via Sig-1Rs to produce its physiological and behavioral effects, although more studies are needed to better characterize this interaction.

13.14 Sigma Receptors and Alcohol

The worldwide consumption of alcohol averages around 6.2 l of pure alcohol per adult per year with the highest overall consumption observed in Eastern Europe and Russia, and with men consuming more alcohol than women [127]. Although most of the adult population worldwide (45 % of men, 66 % of women) abstains from drinking alcohol for most of their lifetime, 76 million adults worldwide are estimated to have alcohol use disorders. Excessive alcohol drinking accounts for a considerable proportion of the global health burden and brings considerable costs to society (both social and health costs) [127, 128].

Alcohol consumption is a major risk factor for disease, with an estimated 4 % of all global deaths and 5 % of global disability-adjusted life-years attributable to alcohol. Excessive alcohol drinking is a necessary cause for approximately 30 different diseases, and a component cause for over 200 diseases; these include cancers, cardiovascular diseases, liver cirrhosis, injuries, and neuropsychiatric disorders [127]. It is estimated that 36.4 % of all neuropsychiatric disability-adjusted life-years are caused by alcohol, and this is mainly due to alcohol use disorders, which are among the most disabling disease categories for the global burden of disease [2].

13.15 Molecular Mechanism

The molecular mechanisms of ethanol action are multiple and not yet entirely understood. Acute ethanol consumption enhances the function of inhibitory neurotransmitters such as gamma-aminobutyric acid (GABA), glycine, and adenosine, and decreases the function of excitatory neurotransmitters such as glutamate and aspartate. In particular, alcohol has been

shown to interact directly with $GABA_A$ receptors, enhance GABAergic currents [129–134], and antagonize N-methyl-D-aspartate glutamate (NMDA) receptors via an allosteric interaction [135, 136].

Although it is often presumed that SigR antagonist-sensitive actions of cocaine and MA reflect direct molecular interactions, their rather low receptorial affinity may suggest that indirect SigR-mediated effects exist for all substances of abuse, including ethanol. A strong body of evidence shows that SigRs mediate some of the locomotor-stimulating, rewarding, and reinforcing effects of ethanol, as described below.

13.16 Sigma Receptors and the Locomotor-Activating and Sedative Effects of Alcohol

Ethanol effects on locomotor activity are a direct function of the dose. At low doses, ethanol exerts locomotor stimulating effects, while at high doses it has sedative effects. The locomotor stimulating properties of ethanol are inferred as a measure of its rewarding properties [137]. The selective Sig-1R antagonist N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine (BD-1047) was shown to dose-dependently attenuate the locomotor-stimulating effects of ethanol in mice [138]. In the same study, the selective Sig-1R agonist PRE-084 failed to affect ethanol-induced locomotion stimulation. Interestingly, neither BD-1047 nor PRE-084 affected locomotor activity *per se* [138]. In line with the previous study, mice lacking the gene encoding for the Sig-1R were found to be less sensitive to the locomotor stimulant effects of ethanol when compared to wild-type (WT) controls [139], strengthening the notion that the Sig-1R is involved in the locomotor-stimulating effects of alcohol. Sig-1R KO mice, on the other hand, did not differ from WT mice in either the latency to lose the righting reflex nor in the time spent sleeping following a high dose of ethanol, suggesting that the Sig-1R does not play a role in the sedative effects of alcohol [139].

13.17 Sigma Receptors and the Rewarding Properties of Alcohol

Alcohol can increase the salience of the contextual stimuli paired with the positive effects of alcohol, which then acquire rewarding properties themselves. An experimental procedure used to evaluate the rewarding properties of a substance is the CPP task. In this paradigm, described in detail above, a compartment equipped with specific visual and textile cues becomes preferred to a second, neutral compartment following repeated pairings with the rewarding substance [38]. Alcohol is able to induce place preference in rodents even though, depending on the specific experimental conditions, this can be technically challenging to obtain [140].

Sig-1R antagonism has been shown to successfully block the expression of the CPP induced by alcohol. Indeed, systemic pretreatment with the selective Sig-1R antagonist BD-1047 administered to mice during conditioning, dose-dependently blocks alcohol-induced acquisition of place preference [138]. In the same study, the authors also showed that the selective Sig-1R agonist 2-(4-morpholino) ethyl 1-phenylcyclohexane-1-carboxylate (PRE-084) produced a dramatic dose-dependent facilitation of ethanol-induced place preference, demonstrating the bidirectionality of the modulation by SigR ligands [138]. These results were confirmed and extended in a study in which BD-1047, administered centrally to mice, blocked not only acquisition, but also expression of ethanol-induced CPP [141]. Interestingly, neither BD-1047 nor PRE-084 produced any change in place preference when administered alone [39, 40, 138].

13.18 Sigma Receptors and Alcohol Drinking

Strong evidence from both human and animal studies supports the overarching hypothesis that SigRs modulate alcohol intake, and propose a role for Sig-1R antagonists as a potential pharmacological therapy for alcohol use disorder.

There is intriguing evidence regarding a functional relationship between alcoholism and polymorphisms in the human Sig-1R gene. Miyatake and colleagues [142] identified three potential candidate polymorphisms: the T-485A, GC-241-240TT, and Gln2Pro polymorphisms, which were significantly associated with alcohol dependence in a Japanese male population. The frequency of the A-485 allele and the TT-241-240/Pro2 haplotype have been shown to be higher in controls relative to alcoholic subjects, suggesting that this polymorphism in the human Sig-1R gene may act as protective factor against alcohol dependence.

Preclinically, a large body of evidence has suggested a bidirectional role for SigRs in modulating alcohol drinking. In rodents, the "two-bottle choice" paradigm is often used to evaluate drinking behavior. In this procedure, rats are provided with continuous access (24 h/day) to two bottles in their home cage, one containing tap water and the other one containing a solution of ethanol, and intake is monitored. In the context of SigR pharmacology, many studies using the two-bottle choice procedure have been performed using the Sardinian alcohol-preferring (sP) rat, a specific rat strain selectively bred to prefer alcohol. sP rats voluntarily drink large quantities of ethanol and show a strong innate preference for ethanol over water, therefore representing a useful tool to study the genetic factors underlying excessive alcohol consumption [143–146].

Sig-1Rs have been demonstrated to exert a key role in the excessive alcohol drinking of sP rats. Sabino and colleagues showed that chronic systemic administration of the selective Sig-1R antagonist BD-1063 dramatically reduced acquisition of alcohol drinking behavior, intake and preference for alcohol in sP rats, without affecting total fluid intake [147]. The same study also found that sP rats, compared to outbred Wistar rats, had innately higher levels of the Sig-1R protein in the nucleus accumbens (NAcc), which were normalized by chronic alcohol consumption [147].

Sig-1R antagonism has also been shown to decrease the maintenance of alcohol drinking in sP rats. Following acute administration, the Sig-1R antagonist NE-100 dramatically reduced excessive ethanol intake and decreased the preference for alcohol, without affecting total fluid intake [148]. In addition, acute NE-100 treatment did not decrease the consumption of sucrose, suggesting that the effect of the drug was selective for alcohol and was not secondary to an overall behavioral deficit [148]. In addition, the alcohol suppressive effect of NE-100 was not due to changes in ethanol pharmacokinetics, as drug treatment did not affect blood alcohol levels when alcohol was administered intragastrically [148].

When injected chronically, daily systemic NE-100 treatment significantly reduced alcohol intake in sP rats [148]. Starting from the sixth treatment day, some tolerance to NE-100's effects was evident, similar to what was observed with an opioid receptor antagonist [149–151].

SigRs are also involved in the increase in alcohol consumption observed when alcohol access is reinstated following a period of deprivation. This "alcohol deprivation effect" has been suggested to be a measure of alcohol craving [152, 153]. sP rats, trained under a two-bottle choice continuous access condition, were forced to abstain from alcohol for one week, and then administered either NE-100 or vehicle before access was renewed. Results showed that the alcohol deprivation effect was fully prevented by pretreatment with the Sig-1R antagonist NE-100 [148].

Recently, it has been shown that Sig-1R KO mice display greater alcohol intake and greater alcohol preference in a two-bottle choice procedure as compared to WT mice [139]. Interestingly, the higher the concentration of alcohol provided, the more pronounced the increase in alcohol intake. Conversely, when mice were tested in a two-bottle choice for either saccharin or quinine, neither the intake of the sweet nor of the bitter solution was changed in Sig-1R KO mice, ruling out that the deletion of the Sig-1R gene resulted in altered taste perception or a general increase in intake of all fluids [139]. Results from this study seem to contradict the overarching hypothesis that Sig-1R activation mediates the effects of alcohol and that Sig-1R antagonism decreases excessive alcohol

drinking [148, 154, 155]. However, the species difference (mice vs. rats) may be responsible for the differential effects observed. In addition, it cannot be ruled out that in whole body KO mice developmental mechanisms play a counteradaptive role and may confound the results obtained.

Two major studies have been pivotal in demonstrating the modulatory role of the SigR in the reinforcing properties of alcohol in rodents. In the first study, the effects of the selective Sig-1R antagonist BD-1063 on alcohol reinforcement were evaluated in two animal models of excessive drinking, namely sP rats and outbred rats made dependent through exposure to chronic intermittent ethanol (CIE) [155]. CIE rats have been shown previously to display increased levels of ethanol self-administration and anxiety-like behavior compared to air exposed rats, as well as increased reward threshold in the intracranial self-stimulation task during withdrawal, making them a valuable tool to study alcohol use disorders [156–159]. The selective Sig-1R antagonist BD-1063 was shown to dose-dependently reduce excessive ethanol self-administration in both sP rats and CIE rats during acute withdrawal [155]. Importantly, BD-1063 did not reduce baseline levels of ethanol self-administration in control rats and did not affect responding for water. In addition, BD-1063 did not reduce sucrose self-administration in sP rats, suggesting that the Sig-1R antagonist effects do not generalize to all reinforcers [155]. In a progressive ratio schedule of reinforcement, BD-1063 was able to reduce the breakpoint for ethanol in sP rats, an objective measure of the subject's motivation [155]. Collectively, these results suggest that Sig-1R hyperactivity may be responsible for the susceptibility to drink excessively.

The results of the second study demonstrated the bidirectionality of the modulation of ethanol drinking exerted by the SigR system. Daily treatment with the SigR agonist 1,3-di-(2-tolyl)guanidine (DTG) was shown to increase ethanol self-administration in sP rats [154]. The increased ethanol drinking in DTG-treated rats resulted in "binge-like" levels of drinking, as rats attained blood alcohol levels that exceeded 80 mg/dL. Importantly, the DTG-induced increase in ethanol intake was reversed by the Sig-1R antagonist BD-1063, confirming that DTG exerted its effects via the Sig-1R subtype. In addition, DTG increased the breakpoint for ethanol in a progressive ratio schedule of reinforcement [154]. Finally, repeated treatment with DTG caused an increase in μ- and δ-opioid receptor gene expression in the ventral tegmental area (VTA) of sP rats, suggesting that SigR agonists may facilitate ethanol's ability to activate the mesolimbic system via the recruitment of the endogenous opioid system in the VTA. These results suggest a key facilitatory role for the SigR in the reinforcing effects of ethanol, and identify a potential mechanism that contributes to excessive drinking.

13.19 Sigma Receptors and Alcohol-Seeking

One of the major issues encountered in the treatment of alcohol addiction is relapse after abstinence. In alcoholic individuals, abstinence is accompanied by craving, a strong desire to engage in alcohol drinking (often termed alcohol-seeking behavior), which in turn can drive relapse [160–162]. Craving can be triggered by a number of different factors including stress, exposure to alcohol (i.e. priming), or exposure to conditioned environmental stimuli previously associated with alcohol (cues).

SigRs have been proposed to be involved in the mechanisms underlying alcohol-seeking behavior induced by priming. It was shown that alcohol-seeking behavior in a place preference paradigm could be reinstated (after extinction) not only by systemic administration of alcohol, but also through central administration of the Sig-1R agonist PRE-084, suggesting that activation of the Sig-1R acts as a "prime" and is sufficient to cross-reinstate alcohol-seeking behavior [141]. The same study also showed that central administration of the Sig-1R antagonist BD-1047 blocked both ethanol-induced reinstatement and the PRE-084 induced cross-reinstatement of ethanol-induced CPP, suggesting that reinstatement of ethanol place preference involves the activation of central Sig-1Rs [141].

As mentioned above, exposure to conditioned stimuli can lead to craving and subsequent resumption of alcohol drinking. In preclinical research, a widely used procedure to assess seeking is cue-induced reinstatement of alcohol-seeking behavior, where reintroducing the conditioned stimuli previously associated with alcohol can reinstate extinguished lever responding. Using this procedure, it was shown that the selective Sig-1R antagonist BD-1047 blocked cue-induced reinstatement of alcohol-seeking behavior [163]. Another classic experimental procedure used to assess alcohol-seeking is the seeking-taking chain in a second order schedule of reinforcement. Here, responding on a seeking lever is maintained not only by the self-administered reinforcer, but also by contingent presentation of the reinforcer-paired stimuli that serve as conditioned reinforcers of the instrumental behavior [161, 164, 165]. Systemic administration of the selective Sig-1R antagonist BD-1063 was shown to reduce alcohol-seeking behavior without affecting responding on the inactive lever [147]. Altogether, these data suggest that the ability of alcohol-associated cues to induce seeking behavior involve the activation of the Sig-1R.

13.20 Sigma Receptors and Cognitive Impairment During Alcohol Withdrawal

Withdrawal from chronic alcohol consumption is characterized by a plethora of physical, motivational, cognitive, and emotional symptoms [166–169]. Withdrawal symptoms can be intense and can develop from several hours to a few days after cessation of heavy and prolonged alcohol use [3, 169, 170]. One of the symptoms associated with withdrawal from chronic alcohol exposure is impairment in cognitive function [166, 167]. In a study conducted by Meunier and colleagues, treatment with either a non-selective Sig-1R agonist (igmesine), or a Sig-1R antagonist (BD-1047), was able to restore cognitive function in a novel object recognition task in mice during withdrawal from chronic alcohol consumption [171]. In addition, mice showed an up-regulation of Sig-1R expression in the hippocampus during withdrawal, which was attenuated following repeated administration of either Sig-1R ligand, suggesting that the increase in hippocampal Sig-1R levels may mediate the cognitive impairment associated with ethanol withdrawal [171].

Using slice electrophysiology, it was shown that withdrawal from chronic intermittent ethanol vapors during adolescence significantly alters long-term potentiation (LTP) in the hippocampus via a Sig-1R related mechanism. One study showed in slices from adolescent CIE-exposed rats that bath-application of the Sig-1R antagonist BD-1047 blocked the characteristic NMDAR-independent LTP, while leaving the normal NMDAR-dependent LTP intact [172]. In addition, the authors showed that SigRs were responsible for the increased presynaptic function in adolescent CIE-exposed animals [172]. In a second study, the same authors found that the Sig-1R antagonist BD-1047 reversed the impairments in CA1 neuronal excitability in slices obtained from adolescent CIE-exposed rats during ethanol withdrawal. These data suggest that acute ethanol withdrawal recruits Sig-1Rs, which in turn act to depress the efficacy of excitatory inputs in triggering action potentials during LTP.

13.21 Sigma Receptors and Hallucinogens

Hallucinogenic substance use was very popular in the 1960s as part of a much broader psychedelic culture and while it has not disappeared, it is now much less widespread. Approximately 2.5 % of the general population reports having used phencyclidine (PCP) at least once and, among all substance use disorders, non-PCP (other) hallucinogen disorders are the rarest [3].

PCP, lysergic acid diethylamide (LSD), and ketamine are commonly abused drugs that induce perceptual distortions including hallucinations and illusions, and disordered thinking such as paranoia. Although the psychedelic effects of hallucinogens generally occur via the serotoner-

gic system and 5-HT2A receptors, certain other behaviors induced by these drugs do not appear to involve monoaminergic systems [173, 174]. In addition, the mechanism of the subjective effects of the different classes of hallucinogenic drugs is not yet entirely clear.

In the context of the Sig-1R system, the SigR agonist DTG has been shown to cross-generalize to the discriminative stimulus effects of the dissociative drug PCP, suggesting commonalities in the discriminative effects of these drugs [175].

Ketamine, which belongs to the class of the dissociative anesthetic agents, is known to produce psychotomimetic effects. It was shown that administration of the Sig-1R antagonist NE-100 caused a rightward shift in the dose-response curve for ketamine discriminative stimulus effects, suggesting that Sig-1Rs are, at least in part, involved in the discriminative stimulus effects of ketamine [176].

N,*N*-dimethyltryptamine (DMT) is a metabolite of the trace amine tryptamine, and is the main ingredient of the hallucinogenic beverage “ayahuasca”. DMT possesses psychedelic effects in humans. In addition, it was shown that DMT is formed in the human and rat brain, making it an endogenous hallucinogen [177–179]. In 2009, it was reported that DMT binds the Sig-1R with moderate affinity (~14 μM) [180]. DMT was also previously found to exert psychomotor stimulating effects in WT mice, but not in Sig-1R KO mice, thereby definitively linking the action of DMT to the Sig-1R [180, 181]. It can therefore be hypothesized that the psychedelic action of DMT might be mediated in part through the Sig-1R, despite the fact that selective Sig-1R agonists do not appear to cause psychotomimetic-like effects in animals [181].

13.22 Concluding Remarks

As reviewed above, it has become increasingly evident that the SigR system is an attractive novel target for treating disorders associated with substance and alcohol use. Since the exact mechanism through which the Sig-1R system influences the actions of psychostimulants and other abused substances has yet to be fully characterized, understanding the mechanism of action of this interaction will undoubtedly advance our understanding of the neurobiological bases of substance use disorders and offer a new therapeutic option for their treatment. Another major obstacle to exploiting the therapeutic promise of this receptor system is a lack of currently available drugs that are selective for SigRs vs. other receptors, as well as for each of the two receptor subtypes. Indeed, whether the Sig-2R exerts the same (or opposite) effects on the actions of abused substances is currently unknown. Therefore, the generation of more selective compounds with enhanced bioavailability, metabolic stability, and low toxicity, may unveil interesting findings and offer substantial therapeutic benefits for the treatment of substance use disorders and associated neurotoxicity.

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14 Stimulation of the Sigma-1 Receptor and the Effects on Neurogenesis and Depressive Behaviors in Mice

Kohji Fukunaga and Shigeki Moriguchi

Abstract

Sigma-1 receptor (Sig-1R) is molecular chaperone regulating calcium efflux from the neuronal endoplasmic reticulum to mitochondria. Recent studies show that Sig-1R stimulation antagonizes depressive-like behaviors in animal models, but molecular mechanisms underlying this effect remain unclear. Here, we focus on the effects of Sig-1R ligands on hippocampal neurogenesis and depressive-like behaviors. Sig-1R stimulation also enhances CaMKII/CaMKIV and protein kinase B (Akt) activities in hippocampus. Therefore, we discuss the fundamental roles of Sig-1R, CaMKII/CaMKIV and protein kinase B (Akt) signaling in amelioration of depressive-like behaviors following Sig-1R stimulation.

Keywords

CaMKII • CaMKIV • BDNF • Neurogenesis • Depression • Sigma-1 receptor

Abbreviations

Akt	protein kinase B
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
CaMKII	calcium/calmodulin-dependent protein kinase II
CaMKIV	calcium/calmodulin-dependent protein kinase IV
CREB	cAMP-responsive element binding protein
DG	dentate gyrus
DHEA	dehydroepiandrosterone
ER/SR	endoplasmic/sacroplasmic reticulum
ERK	extracellular signal-regulated kinase
LTP	long-term potentiation
NMDAR	N-methyl-D-aspartate receptor

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SERCA	sarcoplasmic/endoplasmic Ca^{2+}-ATPase
Sig-1R	sigma-1 receptor
SSRIs	selective serotonin reuptake inhibitors

14.1 Introduction

Sig-1R has been cloned in humans and other species [1–4], and in brain, Sig-1R protein is widely distributed in neurons and glial cells such as astrocytes, and is particularly enriched in prefrontal cortex, hippocampus and striatum [5, 6]. Sig-1R protein is primarily localized in membranes of the endoplasmic/sarcoplasmic reticulum (ER/SR), where it regulates Ca^{2+} signaling through the inositol 1,4,5-triphosphate receptor in close association with mitochondria [7, 8]. Sig-1R stimulation increases release of the neurotransmitters dopamine and glutamate [9, 10]. However, mechanisms underlying these activities remain unclear.

Interestingly, restricted exposure of the hippocampus to X-irradiation blocks DG (dentate gyrus) neurogenesis and compromises the ability of anti-depressants to improve depressive behaviors [11]. Consistent with this observation, in post-mortem analysis of tissues from patients with major depressive disorders, chronic treatment with tricyclic anti-depressants (TCAs) such as imipramine increases the number of neural progenitor cells in the DG [12]. Treatment with selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine or fluvoxamine also improve impaired adult hippocampal neurogenesis in the rodent DG [11, 13]. The observations that these SSRIs and imipramine bind to Sig-1R [14] and that Sig-1R null mice exhibit depressive-like behaviors [15] suggest that Sig-1R stimulation mediates neurogenesis and improvement of depression following treatment with anti-depressants. Indeed, impaired depressive-like behaviors in olfactory bulbectomized (OVX) mice improve following chronic oral administration of dehydroepiandrosterone (DHEA), an endogenous Sig-1R ligand [16, 17].

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) is a serine-threonine protein kinase activated by nuclear Ca^{2+} elevation that catalyzes phosphorylation of the cyclic AMP-responsive element binding protein (CREB) at residue Ser-133 [18, 19]. In rodents, this modification regulates expression of several genes, including *BDNF*, that function in synaptic plasticity [20], learning and memory [21–23], and emotional behaviors [24–26]. CaMKIV is widely distributed in neurons in the anterior cingulate cortex, somatosensory cortex, insular cortex, cerebellum, hippocampus, and amygdala, where it is localized primarily to nuclei [27]. As shown in Fig. 14.1, in mouse hippocampus CaMKIV is expressed in immature neurons positive for PSA-NCAM (a marker of newly generated immature granule cells) and in neurons positive for calbindin, a marker of mature granule cells. CaMKIV is also expressed in radial glia and astrocytes labeled with anti-BLBP (brain lipid binding protein) [28]. Accumulating evidence demonstrates that CaMKIV null mice display deficits in contextual and cued fear conditioning memory [29] and a decrease in anxiety-like behaviors [29, 30]. Furthermore, treatment with the typical SSRI fluoxetine fails to induce DG neurogenesis and does not have an anti-depressive effect in CaMKIV null mice [31].

14.2 Critical Role for Sig1-R in Depression

The depressive-like behaviors shown by Sig-1R null mice [15, 32] are associated with impaired neurogenesis in the hippocampal DG [33]. Sig-1R null male mice show depressive behaviors and reduced hippocampal neurogenesis, phenotypes not seen in female mice [34]. Enhanced estradiol (E2) levels may account for the absence of depressive-like phenotypes in female Sig-1R nulls, as E2 deprivation by ovariectomy in female mice elicits depressive-like behaviors in Sig-1R null mice [34]. E2 administration to male Sig-1R null mice rescues depressive-like behaviors, and src-dependent NMDAR phosphorylation is associated with amelioration of depressive-like behaviors in male hippocampus [34]. These find-

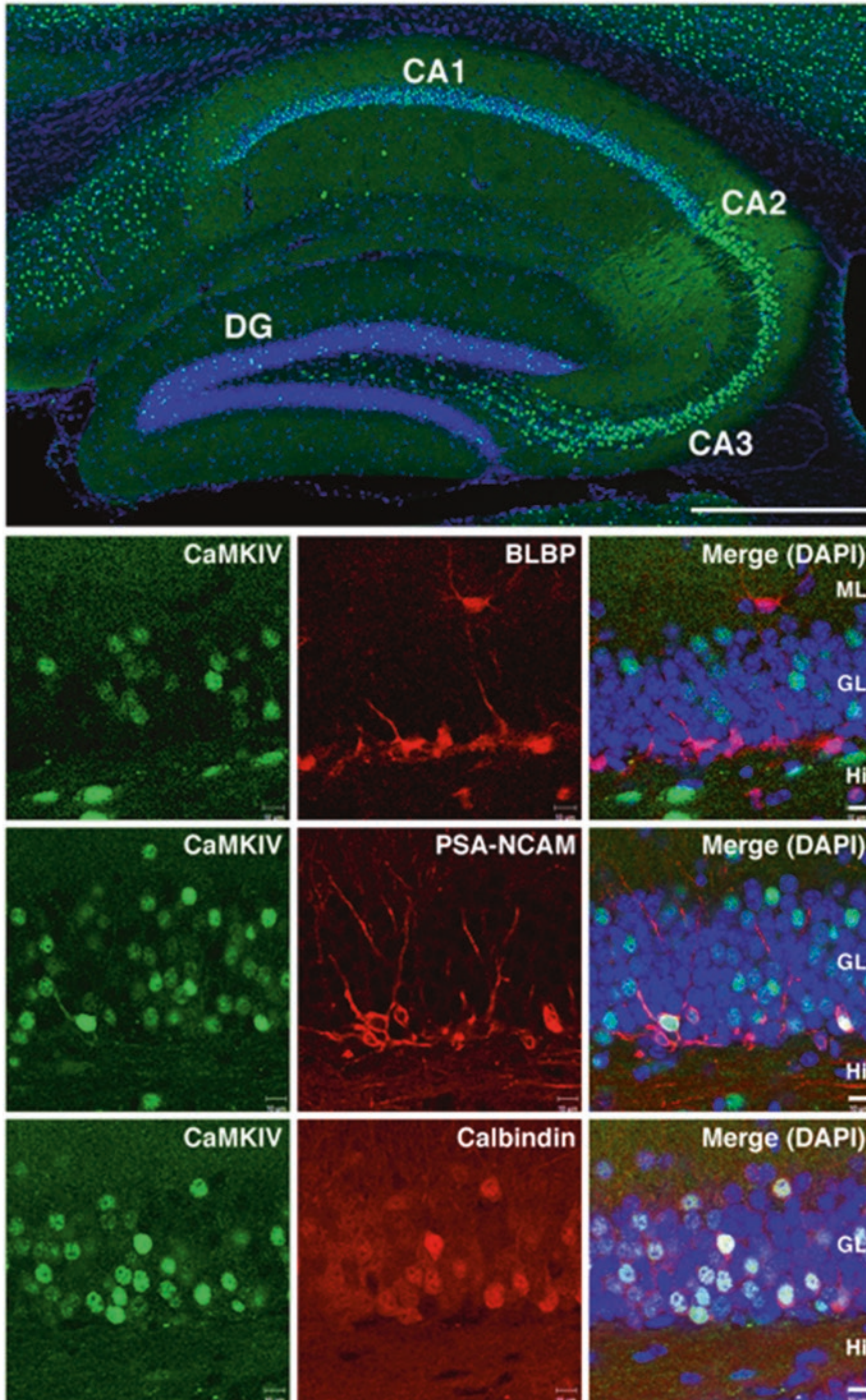

Fig. 14.1 CaMKIV co-localizes with the neuronal markers PSA-NCAM and calbindin but not with the glial marker brain lipid binding protein (BLBP) in the dentate gyrus. Confocal microscopy images showing double immunofluorescence staining of the adult DG for CaMKIV and BLBP, PSA-NCAM or calbindin, as indicated. Merged images show nuclear staining with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue) (Modified from Moriguchi et al. [28])

ings suggest overall that NMDAR activation by Sig-1R mediates E2-induced neurogenesis and amelioration of depressive-like behaviors, either directly or indirectly.

Such phenotypes have been confirmed by pharmacological experiments. Indeed, among antidepressants, fluvoxamine and sertraline show a high affinity for Sig-1R, while fluoxetine, citalopram, and imipramine show low [35]. Specifically, the order of affinity of SSRIs for Sig-1R is: fluvoxamine (K_i = 36 nM) > sertraline (K_i = 57 nM) > fluoxetine (K_i = 120 nM) > citalopram (K_i = 292 nM) > paroxetine (K_i = 1893 nM) [35]. On the other hand, inhibitory constants (*Ki*) for inhibition of serotonin uptake into rat brain are: paroxetine (*Ki* = 0.7 nM) > citalopram (*Ki* = 2.6 nM) > sertraline (*Ki* = 3.4 nM) > fluvoxamine (*Ki* = 6.2 nM) > fluoxetine (*Ki* = 14 nM) [36]. Although Sig-1R is predominantly expressed in the mitochondrion-associated ER membrane (MAM) with the IP_3 receptor, once Sig-1R binds ligand, it translocates to the plasma membrane, activating NMDAR and elevating Ca^{2+} at postsynaptic regions.

Interestingly, Sig-1R levels are relatively decreased in hippocampus of CaMKIV null mice, and fluvoxamine or SA4503 treatment rescues those levels and improves paroxetin-resistant depressive-like behaviors in CaMKIV mutant mice (Fig. 14.2). Sig-1R is highly expressed in astrocytes in the DG subgranular zone, a region stimulated with fluvoxamine or SA4503. SA4503 completely rescues impaired neurogenesis in CaMKIV null mice (Fig. 14.3) [28]. Likewise treatment with fluvoxamine or SA4503, but not paroxetine, also rescues reduced ATP production seen in hippocampus of CaMKIV null mice. This lack of effect by paroxetine suggests that Sig-1R stimulatory action rather than inhibition of serotonin reuptake is critical for fluvaxamine's antidepressive activity. However, lack of amelioration by fluoxetine as reported by Sha et al. [33] cannot be explained by low affinity for Sig-1R. The Sig-1R-specific agonist SA4503 ameliorates impaired adult hippocampal neurogenesis in DG and depressive behaviors in CaMKIV null mice [28]. However, mechanisms underlying depressive behaviors in CaMKIV mice are largely unknown, although reduced CREB/BDNF activity and impaired neurogenesis seen in these mice play a role. More importantly, decreased phosphorylation of CREB, Akt and CaMKII seen in CaMKIV null mice is restored by treatment with fluvoxamine or SA4503.

14.3 CaMKII Activation by Sig-1R Stimulation

It is important to understand how CaMKII is activated by Sig-1R stimulation, as CaMKII autophosphorylation is closely associated with neuronal NMDAR activity. Chronic administration of a Sig-1R agonist is required for CaMKII activation in neurons [28] and Sig-1R activation potentiates NMDAR-mediated responses in neurons [37–41]. For example, Sig-1R stimulation increases the number of NMDARs expressed at the plasma membrane. In rats, 90 minutes after intraperitoneal administration of Sig-1R agonists such as (+)-SKF10, 047, PRE-084 or (+)-pentazocine, synthesis of the NMDAR subunit proteins GluN2A and GluN2B and the postsynaptic density protein 95 (PSD-95) is enhanced hippocampus, effects totally abolished by treatment with the protein synthesis inhibitor anisomycin [41]. Although mechanisms potentially stabilizing newly synthesized NMDARs by Sig-1R remain unclear, direct interaction of Sig-1R with NMDAR has been documented: Sig-1R directly interacts with the GluN1 subunit of NMDAR through its N-terminal region [42]. When Sig-1R-FLAG is coexpressed with either GluN1 or GluN2A in embryonic kidney tsA 201 cells, only GluN1 colocalizes with Sig-1R-FLAG. In addition, the Sig-1R agonist dehydroepiandrosterone (DHEA) stimulates protein kinase C activity and promotes phosphorylation of NMDAR at GluN1 (Ser-896) in olfactory bulbectomized (OBX) mice. Increased NMDAR phosphorylation levels are closely associated with CaMKII activation in OBX mice and reportedly improve memory deficits. DHEA is an abundant, endogenous neuroactive steroid that has anti-amnesic effects through Sig-1R stimulation [43]. Dehydroepiandrosterone sulfate (DHEAS) also stimulates phosphorylation of NMDAR at GluN1 (Ser-896) through

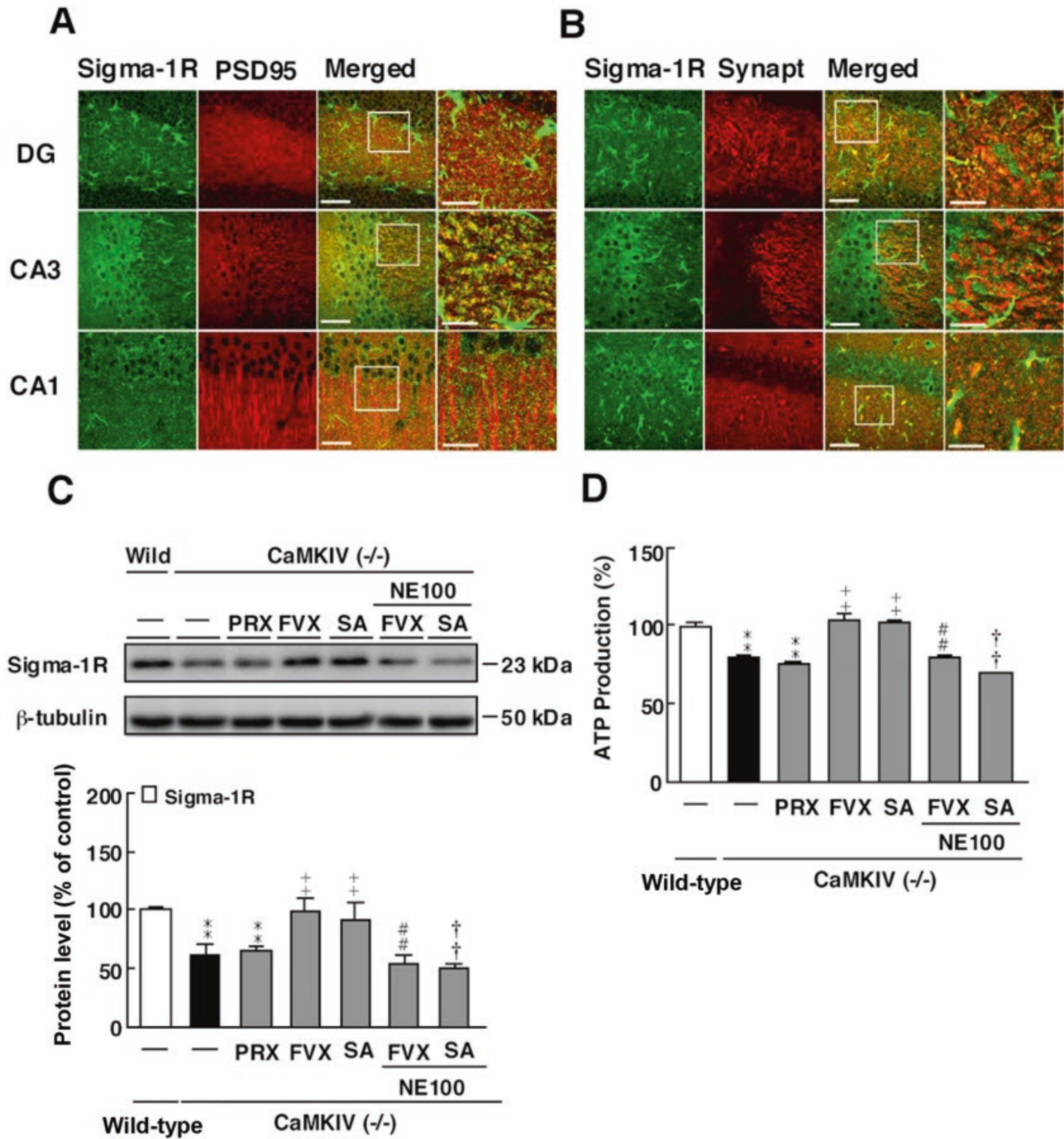

Fig. 14.2 Fluvoxamine or SA4503 treatment but not paroxetine rescues decreased Sig-1R expression and ATP production in the dentate gyrus of CaMKIV null mice. (**a**, **b**) Confocal microscopy images showing double staining for Sig-1R (*green*), PSD95 (**a**) or synaptophysin (**b**) (*red*) and merged images in hippocampal slices. Far right columns show high magnification images of boxed regions in the adjacent image. (**c**) Representative images of immunoblots using antibodies against Sig-1R and quantitative analyses. (**d**) Quantitative analyses of ATP production. Vertical lines show SEM (**, $p < 0.01$ versus wild-type mice. ++, $p < 0.01$ versus CaMKIV null mice. ##, $p < 0.01$ versus fluvoxamine-treated CaMKIV null mice.††, < 0.01 versus SA4503-treated CaMKIV null mice. Modified from Moriguchi et al. [28]

Sig-1R stimulation in spinal cord, an event that mediates NMDA-induced pain behavior in mice [44]. Taken together, Sig-1R promotes stability and intracellular trafficking of NMDAR and increases its phosphorylation through protein kinase C, thereby stimulating CaMKII activity.

Although CaMKIV has been proposed to mediate CREB (Ser-133) phosphorylation,

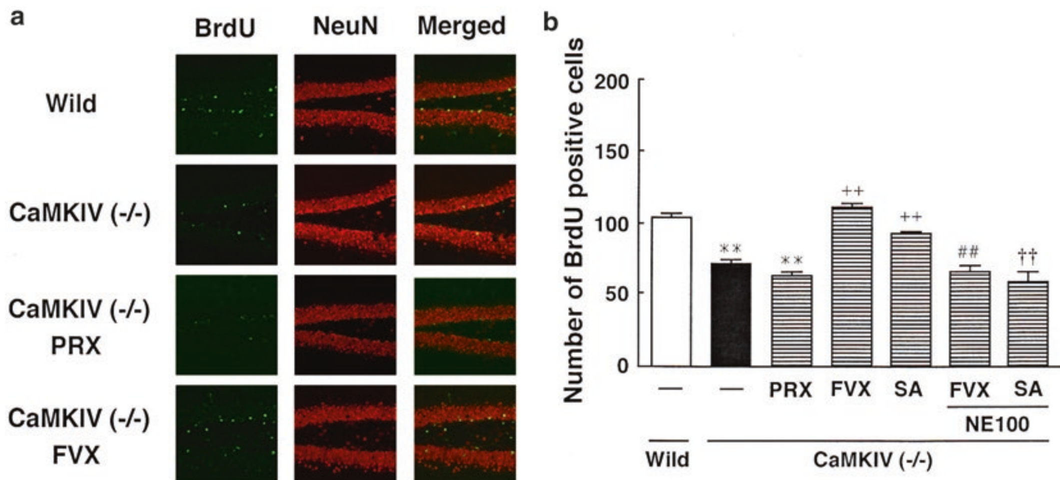

Fig. 14.3 Fluvoxamine or SA4503 but not paroxetine enhances hippocampal neurogenesis in CaMKIV null mice. (**a**) Confocal microscopy images showing double staining for BrdU (*green*), NeuN (*red*) and merged images in hippocampal slices from wild-type mice, CaMKIV null mice, paroxetine-treated CaMKIV null mice, fluvoxamine-treated CaMKIV null mice, SA4503-treated CaMKIV null mice, NE100 (Sig-1R antagonist) plus fluvoxamine-treated CaMKIV null mice and NE100 plus SA4503-treated CaMKIV null mice. Mice were injected with BrdU on the first day of drug treatment and then for 5 consecutive days during the 2 weeks of drug treatment. Mice were treated with paroxetine, fluvoxamine, or SA4503 treatments for 2 weeks (n = 8). (**b**) Quantitative analyses of the number of BrdU/NeuN double-positive cells in the DG (n = 8). Vertical lines show SEM. **, $p < 0.01$ versus wild-type mice. $^{++}$, $p < 0.01$ versus CaMKIV null mice. ##, $p < 0.01$ versus fluvoxamine-treated CaMKIV null mice. ††, <0.01 versus SA4503-treated CaMKIV null mice (Modified from Moriguchi et al. [28])

CaMKII primarily accounts for CREB phosphorylation and BDNF expression in CaMKIV null mice, an idea confirmed by the fact that expression of BDNF mRNA containing exons I or IV is upregulated in the DG of CaMKIV null mice by Sig-1R stimulation. Likewise, Sun et al. [45] reported that unlike CaMKIV, CaMKII regulates CREB activity through phosphorylation of CREB at residue Ser-142 (in addition to Ser-133). CaMKII overexpression increases levels of BDNF transcripts containing exon IV in NG108–15 cells [46]. NMDAR stimulation [9, 47, 48] and increases in ATP production [8] by Sig-1R ligands are two of the mechanisms underlying CaMKII activation in neurons. Increased ATP production enhances Ca^{2+} storage in the ER by stimulating the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) pump, which can promote Ca^{2+}-induced Ca^{2+}-release from the ER and in turn activate neuronal CaMKII activity. The observation of depression-like behaviors in CaMKIV null mice is important, as those behaviors are closely associated with decreased neurogenesis in the hippocampal DG, and CaMKIV is expressed highly in pyramidal neurons in both CA1 and CA3 regions and in DG granule cells [28]. Like CaMKIV null mice, CaMKIIα heterozygous knockout mice show increased numbers of immature granule cells in the hippocampal DG and a decreased number of mature granule cells [49]. Moreover, analysis proliferation by BrdU incorporation shows that the number of BrdU-positive cells slightly increases in CaMKIIα heterozygous knockout mice [49]. Thus, both CaMKIV and CaMKIIα likely function in proliferation and/or maturation of granule cells in the mouse DG.

14.4 Sig1-R Plays a Critical Role in BDNF Expression

Enhanced adult hippocampal neurogenesis is associated with activation of both PI3K/Akt [17, 50, 51] and CREB/BDNF pathways [17, 50]. Both pathways are essential for neuronal prolif-

eration and maturation [52], and their activation by Sig-1R agonists may antagonize depressive behaviors. For example, stimulation of Sig-1R by fluvoxamine or SA4503 markedly activates PI3K/Akt and CREB/BDNF signaling in DG of CaMKIV null mice. Akt activation by fluvoxamine and SA4503 is also associated with tyrosine kinase signaling that promotes NMDAR activation [53] or NMDAR-dependent BDNF expression though CaMKII signaling [54]. In addition to CaMKII-dependent BDNF expression, chaperone activity is crucial for BDNF maturation and release of BDNF from neurons [55, 56]. In rat neuroblastoma B104 cells, SA4503 treatment increases the secretion of BDNF (pro plus mature BDNF) [55]. Fujimoto et al. [55] have proposed that chronic treatment with SA4503 potentiates post-translational processing of BDNF by activating Sig-1R chaperone activity at the ER membrane.

In addition, a link between Akt and CREB activities has been demonstrated in neural progenitor cells stimulated by fibroblast growth factor-2 (FGF-2), a factor is essential for proliferation of hippocampal progenitors [57]. FGF-2 and insulin-like growth factor-1 (IGF-1) also reportedly enhance proliferation of adult hippocampal neural progenitors [57]. Both mitogens stimulate Akt signaling [57]. In addition, conditional knockout of CREB in mice impairs in vivo proliferation of hippocampal neural progenitors [58]. Although the source of hippocampal FGF-2 and IGF-1 has not been defined, both mitogens are likely derived from astrocytes, based on studies of Shetty et al. [59]. In this context, our observation of immunohistochemical localization of Sig-1R in hippocampal astrocytes is particularly relevant. Cao et al. [60], using IP_3 receptor type 2 transgenic mice, reported that ATP release from astrocytes is critical for anti-depressants to be effective. CaMKIV is not expressed astrocytes and co-localizes with PSA-NCAM and calbindin but not with BLBP in the DG [28]. We confirmed that CaMKIV is expressed in differentiating and mature dentate granule cells but not in neural stem cells or glial cells. Since Sig-1R is highly expressed in astrocytes of the subgranular zone and postsynaptically in CA1 and CA3 regions and its stimulation promotes hippocampal ATP production, Sig-1R stimulation of both astrocytes and postsynaptic neurons likely mediates Sig-1R stimulation-induced neurogenesis. A model of Sig-1R function in both neurogenesis and regulation of BDNF expression is shown in Fig. 14.4. Sig-1R stimulation by fluvoxamine or SA4503 promotes NMDAR function, increasing CaMKII activity. This in turn potentiates LTP through AMPAR phosphorylation and BDNF expression via CREB phosphorylation, even in the absence of CaMKIV activity. BDNF expression promotes increased Akt phosphorylation and neurogenesis. Sig-1R stimulation by fluvoxamine or SA4503 also enhances ATP production by enhancing mitochondrial Ca^{2+} entry. All of these activities likely antagonize depressive-like behaviors in rodent models.

14.5 Sig-1R Plays a Critical Role in Heart and Other Diseases

Depression is associated with substantial risk of developing heart failure and is independently associated with increased cardiovascular morbidity and mortality. Likewise, cardiovascular disease can lead severe depression. Thus, SSRI therapy has been strongly recommended to reduce cardiovascular disease-induced morbidity and mortality. We recently observed very high expression of Sig-1R in rat heart tissue [61] and determined that in rodent heart, the receptor is a direct target of SSRIs [62] and DHEA [63] in eliciting cardioprotection in both pressure overload (PO)-induced and transverse aortic constriction (TAC)-induced myocardial hypertrophy models. Our findings suggest that SSRIs such as fluvoxamine protect against PO- and TAC-induced cardiac dysfunction by upregulating Sig-1R expression and stimulating receptor-mediated Akt-eNOS signaling [63]. In addition, myocardial infarction with aortic banding elicits depressive-like behaviors in mice [64, 65]. Intracerebroventricular injection of the Sig-1R agonist PRE084 in myocardial infarction mice improved both depressive behaviors and cardiac dysfunction, with lowered sympathetic activity

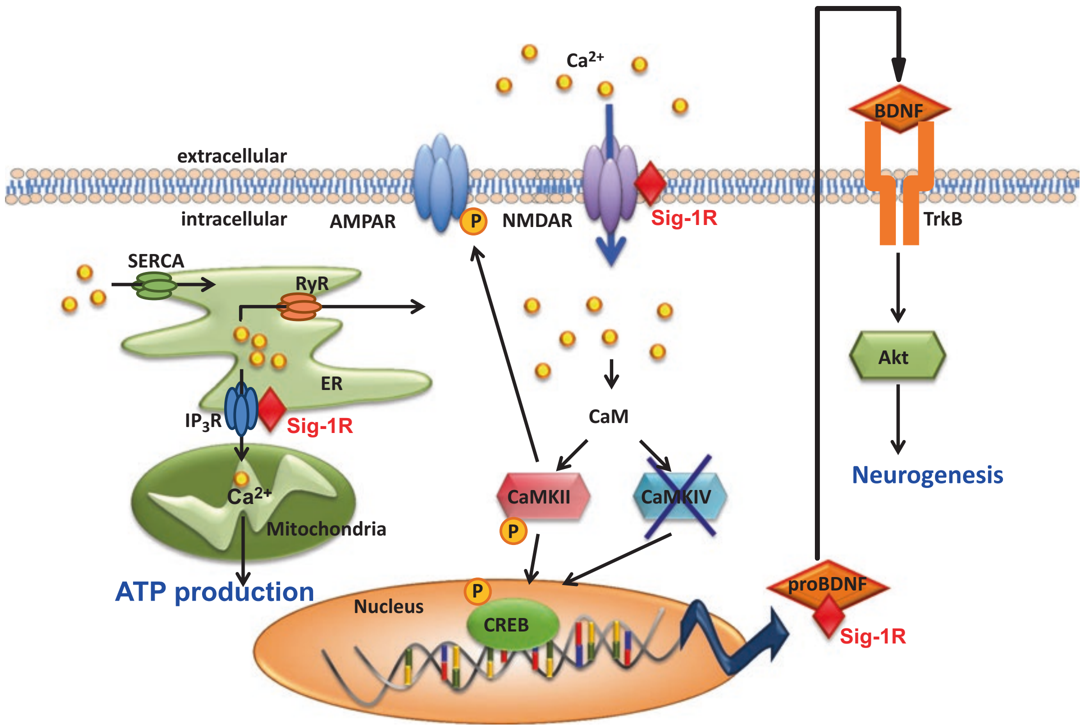

Fig. 14.4 Schematic representation of altered adult hippocampal neurogenesis in the DG. Sig-1R stimulation increases intracellular calcium mobilization through NMDARs in the plasma membrane or through the ER/SR via mitochondrial ATP production. Increased intracellular calcium increases CaMKII autophosphorylation and promotes CREB (Ser-133) phosphorylation and BDNF expression, which in turn increases Akt phosphorylation and promotes adult hippocampal neurogenesis (Modified from Moriguchi et al. [28])

and recovery of Sig-1R expression in brain. Similarly, loss of Sig-1R activity mediates depressive-like behaviors in streptozotocin-induced diabetic rats [66]. The hypothalamic-pituitary-adrenal axis likely functions in perturbed central nervous system (CNS) activity mediated by Sig-1R loss in heart failure and diabetes. As yet, potential inflammatory cytokines or hormones that antagonize CNS Sig-1R signaling have not been identified. However, amelioration of depressive-like behaviors by Sig-1R agonists is particularly important for clinical therapeutics. In addition, the pathophysiological relevance of Sig-1R-mediated changes in ATP production remains unclear. To resolve the question, future studies should focus on development of the specific Sig-1R ligands useful in clinic settings.

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Role of σ_1 Receptors in Learning and Memory and Alzheimer's Disease-Type Dementia

15

Tangui Maurice and Nino Goguadze

Abstract

The present chapter will review the role of σ_1 receptor in learning and memory and neuroprotection, against Alzheimer's type dementia. σ_1 Receptor agonists have been tested in a variety of pharmacological and pathological models of learning impairments in rodents these last past 20 years. Their anti-amnesic effects have been explained by the wide-range modulatory role of σ_1 receptors on Ca^{2+} mobilizations, neurotransmitter responses, and particularly glutamate and acetylcholine systems, and neurotrophic factors. Recent observations from genetic and pharmacological studies have shown that σ_1 receptor can also be targeted in neurodegenerative diseases, and particularly Alzheimer's disease. Several compounds, acting partly through the σ_1 receptor, have showed effective neuroprotection in transgenic mouse models of Alzheimer's disease. We will review the data and discuss the possible mechanisms of action, particularly focusing on oxidative stress and mitochondrial integrity, trophic factors and a novel hypothesis suggesting a functional interaction between the σ_1 receptor and α_7 nicotinic acetylcholine receptor. Finally, we will discuss the pharmacological peculiarities of non-selective

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σ_1 receptor ligands, now developed as neuroprotectants in Alzheimer's disease, and positive modulators, recently described and that showed efficacy against learning and memory deficits.

Keywords
Sigma-1 receptor • Learning and memory • Neuroprotection • Alzheimer's disease • Oxidative stress • Positive allosteric modulators

15.1 The σ_1 Receptor Is a Key Component of Brain and Cellular Plasticity

The σ_1 receptor is a member of a family of membrane-associated proteins, found throughout the body and widely distributed in neurons, astrocytes, oligodendrocytes and microglia within the central nervous system. As a single 25 kD polypeptide and chaperone protein, it is tightly expressed at mitochondria-associated endoplasmic reticulum (ER) membranes (MAM) and plasma membranes [1], bound to the ceramide-enriched microdomain, and also called lipid rafts, in complex with the glucose-related protein 78/binding immunoglobulin protein (GRP78/BiP). The σ_1 receptor has the peculiarity for a chaperone protein, to bind and be activated/inactivated by diverse classes of pharmacological compounds, including antipsychotics, opioids, antidepressants, antagonists of muscarinic, D2 dopamine and N-methyl-D-aspartate (NMDA) receptor ligands, monoamine transporters inhibitors, selective serotonin reuptake inhibitors and monoamine oxidase inhibitors. Although certain endogenous ligands have been identified interacting with the σ_1 receptor, such as neuro(active)steroids, neuropeptides, or trace amines, the precise nature of its high-affinity endogenous ligand is still unclear [2].

In resting condition, the σ_1 receptor resides with the ER-resident chaperone BiP, however, under ER stress or via agonist stimulation σ_1 receptor dissociates from BiP and binds inositol 1,4,5-trisphosphate (IP_3) receptor, enhancing calcium entry into mitochondria [1]. Increase in redox reactions and ATP production by regulating Ca^{2+}-dependent enzymes in the tricarboxylic acid cycle is evoked by Ca^{2+} entry into mitochondria, whereas mitochondrial Ca^{2+} overload leads to apoptosis [3, 4]. Shioda et al. [5] identified a truncated isoform of σ_1 receptor, a novel splice variant, defined as a short form of σ_1 receptor (σ_1SR). Interestingly, σ_1SR did not form a complex with IP_3 receptor, but had the ability to bind to σ_1 receptor and its overexpression reduced mitochondrial Ca^{2+} uptake in response to IP_3 receptor driven stimuli. Indeed, σ_1SR suppresses ATP production following ER stress, enhancing cell death [5]. The σ_1 receptor is able to modulate a variety of intracellular signal transduction pathways through protein-protein interactions. In addition, after activation, the σ_1 receptor has the ability to translocate to other cell compartments and binds to different membrane proteins, including ion channels, kinases, G-protein coupled receptors, or trophic factor receptors. The σ_1 receptor has been shown to interact with K^+, Ca^{2+}, Cl^-, and Na^+ channels [6]. Among the physiological impact on brain plasticity and memory, the modulation of voltage-gated ion channels [7, 8], which are mainly involved in the initiation and shaping of action potentials and global cell excitability [9, 10], or the modulation of NMDA-induced neuronal firing in the CA3 region of the hippocampus [11], for instance, are the most pertinent actions. It was also reported that, in neurons, σ_1 receptor activation inhibited the recruitment and coupling of the Ca^{2+}-dependent nitric oxide (NO) synthase (nNOS) to postsynaptic density protein-95 (PSD95) [12, 13].

The σ_1 receptor is able to shape cellular plasticity in neuronal cells by directly modulating the activity of pleiotropic transcription factors such as nuclear factor κB (NFκB), cyclic adenosine monophosphate (cAMP) response element-binding (CREB) protein and c-fos. These transcrip-

tion factors are involved in the modulation of pro- and anti- inflammatory genes as well as cell death and survival [14]. In the plasma membrane, the σ_1 receptor may directly control the dendritic spine arborization by increasing Rac-GTP through regulation of the level of reactive oxygen species (ROS) [15] and a direct interaction between the σ_1 receptor and Rac1-GTPase was described in brain mitochondria [16]. The σ_1 receptors therefore appear to be a unique class of proteins influencing and participating in a wide range of biological pathways, including Ca^{2+} signaling at the ER, controlling several families of ion channels at plasma membrane and MAM, thus maintaining ER-mitochondria exchanges, and modulating transcription factors. The σ_1 receptor-mediated neuromodulation, affecting several cellular pathways has an important role on brain plasticity and particularly learning and memory processes.

15.2 The σ_1 Receptor Plays a Role in Learning and Memory Processes

The impact of σ_1 receptors in learning and memory processes has been known since the early 90's and numerous data have been accumulated on the potential efficacy of σ_1 receptor agonists as anti-amnesic drugs. Availability of the constitutive σ_1 protein knockout (σ_1 KO) mice in the mid-2000's allowed to directly analyze the impact of σ_1 gene deletion on learning abilities in mice [17]. Two-month old male σ_1 KO mice showed signs of anxiety in the open-field, passive avoidance and elevated plus-maze tests and an enhanced response in the forced swimming test. The σ_1 gene invalidation therefore affected stress or anxiety response, but memory responses were unchanged. Female σ_1 KO mice showed deficits in spontaneous alternation or water-maze learning. Twelve-month old, heterozygous σ_1 KO female mice showed deficits in alternation and homozygous σ_1 KO female mice showed deficits in avoidance escape latency. So, σ_1 KO female mice showed clear learning impairments that amplified with age. Both 2- and 14-month old female σ_1 KO mice showed decreased plasma levels of 17β-estradiol and a supplementation treatment with the hormone reversed the spatial memory deficits in young and aged mice [17], suggesting that the invalidation of the σ_1 gene had a developmental impact on steroid tonus.

The main indications that σ_1 receptor activity could impact learning and memory came from studies showing that σ_1 receptor ligands, administered systematically to mice or rats undergoing several different behavioral analyses, improve their learning ability in pathological conditions. Since the initial reports of Earley et al. [18] and Maurice et al. [19], numerous evidences have accumulated during the last two decades showing that, if σ_1 receptor agonists are not promnesic compounds per se, they are effective anti-amnesic drugs in a variety of pharmacological or pathological models of amnesia. Earley et al. [18] described for the first time that the σ_1 receptor agonists igmesine, (+)-3-PPP and DTG alleviated the learning impairment in mice submitted to a passive avoidance test induced by the non-selective muscarinic acetylcholine (mACh) receptor antagonist scopolamine. This observation was reproduced by the group of Matsuno et al. [20–24], who notably used cutamesine (SA4503), a selective σ_1 receptor agonist, and correlated the behavioral improvement with analyses of the σ_1 receptor-induced ACh release in the hippocampus and cortex. The result was then validated by several groups [25–31]. Maurice et al. [19, 32] tested several σ_1 receptor ligands, including DTG or (+)-SKF-10,047, in mice treated with the NMDA receptor non-competitive antagonist dizocilpine ((+)-MK-801). The σ_1 receptor agonists alleviated the learning deficits, measured in the spontaneous alternation or passive avoidance paradigms. These data were also later reproduced by other groups [33–35]. These pioneering studies demonstrated in a coherent manner that σ_1 receptor agonists are anti-amnesic drugs in pharmacological models of amnesia involving either cholinergic or glutamatergic blockade. The compounds however, did not impact learning abilities alone, i.e., σ_1 receptor agonists did not improve memory in control animals. Moreover,

all the studies consistently showed that σ_1 receptor antagonists, including BMY-14,802, haloperidol, BD-1047, NE-100, blocked σ_1 receptor agonist-mediated beneficial effects but without impairing learning or causing amnesia by themselves in control animals. The beneficial effect of the σ_1 receptor agonists against learning deficits was bell-shaped, defining an optimal dose-range depending on the species, the behavioral test and the amnesia model used (for reviews, see [36–39]).

σ_1 Receptor agonists have been finally tested in several pharmacological models of amnesia induced by *p*-chloroamphetamine, phencyclidine, L-NAME, nimodipine, or an amyloid-β (Aβ) peptide [20, 40–46]. Moreover, the beneficial effects of σ_1 receptor agonists on memory impairments have been explored in several lesional models, including ibotenic acid forebrain lesion, *nucleus basalis* lesions, 192 IgG-saporin induced lesions, repeated exposure to carbon monoxide (CO) or trimethyltin intoxication [23, 47–53], and developmental deficits, like prenatal stress or in utero exposure to addictive doses of cocaine [54, 55]. Finally, a couple of studies addressed the beneficial effect of σ_1 receptor agonists against ageing-related learning deficits, first, in a model of accelerated aging, the senescence-accelerated mouse (SAM), and, second, in aged rats [56, 57]. The σ_1 receptor drugs consistently showed effective beneficial effects in rodents, using numerous behavioral assessment to assess short- and long-term memory, spatial and non-spatial memory or recognition memory. These assessments included spontaneous alternation, passive and active avoidances, place learning in the water-maze, novel object recognition, three-panel runway, and radial arm maze. Modulation of the σ_1 receptor therefore involves a wide-range mechanism of action, affecting all phases of mnesic processes, namely learning, consolidation, retrieval, and forgetting [58].

Curiously, few studies addressed the anatomical localization and function of σ_1 receptor in brain structures involved in memory, notably by anatomical lesions. High density of protein has however been identified in pertinent areas, like the hippocampal formation, the frontal, entorhinal, pyriform cortex and basal ganglia structures [59], substantiating the effect of the compounds. Moreover, changes of σ_1 receptor expression during ageing has been examined, historically by autoradiographic and binding techniques and more recently by positon-emission tomography (PET). In the rodent brain, σ_1 receptor density was generally found to be preserved during aging. The first study used ^{3}H–haloperidol, in combination with unlabeled spiperone to block D2 dopamine receptors. The σ_1 receptor density in the brain of Fisher-344 rats was found to be unaltered between postnatal day 1 and 12 months of age [60]. In C57Bl/6 mice, no difference was observed in σ_1 receptor density between 2- and 24-month old animals, either at the mRNA nor protein level [61]. Similarly, in the SAM model, no differences in σ_1 receptor density of various brain regions were observed between 6-, 9- and 12-month-old SAMP/8 senescence-prone mice and control SAMR/1 senescence-resistant mice, in terms of mRNA or protein level, measured by PCR, histochemistry and ^{3}H(+)-SKF-10,047 binding [62]. In the rat brain, the binding of several σ_1 receptor radioligand, ^{3}H–SA4503, ^{3}H-(+)pentazocine and ^{3}H–DTG, has been examined in 1.5-, 6-, 12- and 24-month-old Fisher-344 rats [63]. The number of binding sites increased with ageing, but the binding affinity of all ligands was decreased. The authors therefore suggested that increases in receptor density compensated for a reduced affinity of the receptor proteins to the radiotracers [63]. PET analyses, using the radiotracer ^{11}C–SA4503, showed that the binding potential to σ_1 receptors increased in the brain of aged monkeys as compared with young adult [64], but was unchanged in the human brain during healthy ageing [65]. These observations contrasted strikingly with the age-dependent loss of cholinergic, glutamatergic and dopaminergic receptors, which occurs in primates and humans during ageing, and confirmed the pertinence to target σ_1 receptors in age-related cognitive deficits.

15.3 Mechanism of Action of σ_1 Receptor Ligands in Memory: Modulation of Neurotransmitters and Trophic Factors

The impact of σ_1 receptor ligands on memory is usually explained by the direct neuromodulatory role on σ_1 receptor on several neurotransmitter systems and signaling pathways involved in the encoding or retrieval of memory. Mainly, σ_1 receptor activity enhances glutamatergic synapses, cholinergic synapses and the effects of trophic factors, particularly the brain-derived neurotrophic factor (BDNF), whose role in memory is known to be crucial.

15.3.1 Glutamatergic Systems

The σ_1 receptor activity modulates positively NMDA receptors. It is known since the early 90's that NMDA-induced firing and monoamine release in the hippocampus can be enhanced by selective σ_1 receptor agonists at very low dose [11, 66, 67]. σ_1 Receptor activity also impact long-term potentiation (LTP). For instance, chronically administered dehydroepiandrosterone sulfate (DHEAS), a σ_1 receptor agonist neuroactive steroid, significantly facilitated the induction of frequency-dependent LTP in rat hippocampal CA1 pyramidal cells, in a haloperidol and NE-100-sensitive manner [68]. The steroid did not alter presynaptic glutamate release in response to both single pulse and tetanic stimulation, suggesting that certain alterations happened in postsynaptic neurons [68]. The effect was proposed to involve Src-dependent NMDA receptor signaling and direct regulation of the tyrosine phosphorylation of the NMDA receptor subunit 2B (NR2B), particularly since tyrosine phosphorylation of NR2B was significantly decreased after reversible forebrain ischemia in rats and improved after repetitive administration of DHEAS, whereas that of NR1 had no obvious change [69]. The mechanism regulating the σ_1 receptor interaction with NMDA receptors also involved regulation of a small conductance Ca^{2+}-activated K^+ current (SK channels). Martina et al. [70] reported, using patch-clamp whole-cell recordings in CA1 pyramidal cells of rat hippocampus, that (+)-pentazocine potentiated NMDA receptor responses and LTP by preventing SK channels, an entity known to shunt NMDA receptor responses, to open. Very recently, the same authors reported that the selective σ_1 receptor agonists (+)-SKF-10,047, PRE-084, and (+)-pentazocine increased the expression of NR2A and NR2B subunits, as well as PSD95, in the rat hippocampus. The σ_1 receptor activation led to an increased interaction between NR2 subunits and σ_1 receptors and mediated trafficking of NMDA receptors to the cell surface [71]. These observations illustrated the major role played by σ_1 receptor activation in NMDA receptor-mediated activity and trafficking, key processes in learning and memory.

15.3.2 Cholinergic Systems

The cholinergic neurotransmission is crucial in memory and cognitive function, not only for learning, but also in the consolidation and retrieval phases of the mnesic processes. Cholinergic basal forebrain neurons in the *nucleus basalis magnocellularis* innervate the cerebral cortex, amygdaloid complex and hippocampus and are essential for memory formation [72]. σ_1 Receptor activity affects cholinergic pathways either indirectly, notably through the modulation of NMDA receptors, in the hippocampus, septum and cortex, but also directly. First, σ_1 receptor agonists are potent modulators of ACh release, both in vitro and in vivo. (+)-SKF-10,047, igmesine or cutamesine potentiated the KCl-evoked release of ^{3}H–ACh from rat hippocampal slices [73, 74]. The σ_1 agonists (+)-SKF-10,047, (+)-3-PPP, (±)-pentazocine, DTG and cutamesine acutely and dose-dependently increased extracellular ACh levels in the frontal cortex and hippocampus, using in vivo microdialysis in freely moving rats [21, 40, 75]. Interestingly, the absence of effect of cutamesine on electrically evoked release of

^{3}H–ACh in vitro or extracellular ACh levels in vivo in the striatum [74, 76] suggested that the σ_1 receptor effect on ACh release could be region-specific and this differential regulation pertinent to explain why σ_1 receptor drugs do not display some undesired side effects which are frequently seen after administration of acetylcholinesterase (AChE) inhibitors. The mechanism by σ_1 receptor ligand induce ACh release is directly related to the σ_1 receptor-mediated modulation of Ca^{2+} mobilization, via IP_3 receptor-gated pools and voltage-gated K^+ and Ca^{2+} channels [77, 78].

15.3.3 Trophic Factors

Trophic factors play prominent roles during brain development and also in the mature nervous system to ensure effective plasticity. BDNF, through activation of its selective receptor TrkB, and nerve-growth factor (NGF), through TrkA activation, have been shown to play important roles in learning and memory by directly regulating glutamate or ACh syntheses and releases. They exert neurotrophic actions on cholinergic neurons of the basal forebrain *nuclei* and are synthesized by hippocampal and cortical neurons that are located in the projection field of the basal forebrain cholinergic neurons. Their release and the regulation of their synthesis is dependent on specific neurotransmitter systems, the refined tuning of synaptic functions. BDNF particularly is a regulator of dendrite sprouting, axon branching and activity-dependent refinement of synapses [79] with direct implications in memory functions (for a recent review, see [80]), and possible therapeutic consequences for the treatment of Alzheimer's disease [81]. Several ligands acting at the σ_1 receptor, either selectively (E-5842, cutamesine, PRE-084, DTG) or non-selectively (fluvoxamine, captodiamine) have been reported to modulate BDNF expression [82–87]. In particular, Xu et al. [87] described that PRE-084 and DTG ameliorated learning impairments and prevented the decline of synaptic proteins and BDNF expression in the hippocampus of mice submitted to bilateral carotid artery occlusion. The σ_1 receptor ligands up-regulated the level of NR2A, calcium/calmodulin-dependent protein kinase type IV (CaMKIV) and CREB-specific co-activator transducer of regulated CREB activity 1 (TORC1), confirming that σ_1 receptor activation increased the expression of BDNF, through the NR2A/CaMKIV/TORC1 pathway. An interesting study by Yagasaki et al. [88] was based on the fact that BDNF signaling also contributes to the effects of antidepressants. They examined the antidepressant effects on BDNF signaling through the PLC-γ/IP_3/Ca^{2+} pathway and its modulation by σ_1 receptors. The BDNF-stimulated PLC-γ activation and consequent increase in $[Ca^{2+}]_i$ and BDNF-induced glutamate release were potentiated by imipramine or fluvoxamine, in a BD1047-sensitive manner. Overexpression of σ_1 protein per se enhanced BDNF-induced PLC-γ activation and glutamate release [88]. These data clearly illustrated that the σ_1 receptor plays an important role in BDNF signaling leading to glutamate release. A similar regulation is expected in learning and memory processes, since they rely on the same hippocampal pathways.

15.4 The σ_1 Receptor Is an Endogenous Protection System in Alzheimer's Disease and a Target for Neuroprotective Therapies

Alzheimer's disease (AD) is one of the most common forms of dementia in the world. According to the World Health Organization, 47.5 million people have dementia worldwide, and they are 7.7 million new cases per year. By 2025, the number of peoples aged 65 and older with Alzheimer's disease is estimated to reach 7.1 million in the United States (data from the Alzheimer's Association). AD is clinically characterized by progressive cognitive decline and pathologically by the presence of extracellular senile plaques composed primarily of amyloid-β peptide (Aβ) and intracellular accumulation of neurofibrillary tangles made up mainly of

hyperphosphorylated Tau protein [89]. Currently, an autopsy or brain biopsy is the only way to make a definitive diagnosis of AD. In clinical practice, the diagnosis is usually made on the basis of the history and findings on Mental Status Examination. Symptomatic therapies are the only treatments available for AD. The standard medical treatments include cholinesterase inhibitors and a non-competitive NMDA receptor antagonist. Psychotropic medications are used as a treatment of the secondary symptoms of AD, such as depression, agitation, and sleep disorders. Understanding the pathophysiology of AD is important for developing effective treatment strategies.

It has been proposed that σ_1 receptors actively participate in the endogenous cellular defense against neurodegenerative disorders, such as AD, amyotrophic lateral sclerosis or Parkinson's disease, by regulating oxidative stress, apoptosis, Ca^{2+} signaling at MAMs, and mitochondrial metabolism. Indeed, neuroimaging studies revealed that σ_1 receptors are present in lower density in brains from AD patients relative to the brains of healthy individuals [90]. It has also been seen that certain polymorphisms of the σ_1 gene, especially when present alongside the known AD risk factor apolipoprotein ε4 (APOE ε4), are linked to the onset of AD neurodegeneration [91]. These observations give a rationale for studying actively σ_1 receptors in AD as a promising therapeutic target.

15.4.1 σ_1 Polymorphisms in AD

Several genetic studies have suggested that the σ_1 gene may be involved in the susceptibility to AD. Two polymorphisms of the σ_1 gene, GC-241-240TT and Q2P have been identified in only two haplotypes: GC-241-240Q2 and TT-241-240P2. When analyzed in a Japanese sample of AD patients and control subjects, the TT-241-240P2 haplotype, which decreases expression of the gene, was found to have a protective role against susceptibility to AD [92]. In a group of Polish patients with late-onset AD, patients with mild cognitive impairment and a control group, no significant differences for the σ_1 allele, genotype, haplotype, and diplotype distributions were reported between the studied groups [93]. In a population of late-onset AD Hungarian patients and ethnically matched elderly control individuals, it was observed, on the contrary, an association between the TT-241-240P2 variant and the risk for developing AD. Particularly, a potential modest interaction effect of the co-presence of the TT-P haplotype with APOE ε4 allele on the risk for AD was suggested [91]. Finally, another study showed that σ_1 protein 2P variant and APOE ε4 allele interact to influence AD severity across ethnic populations. Based on an Australian and Chinese cohorts, a significant genetic interaction was observed between the APOE ε4 allele and σ_1 protein 2P carriers in both populations, i.e., the σ_1 protein 2P variant had increased cognitive dysfunction and more advanced stages of NFT in APOE non-ε4 allele carriers [94]. A consensus did not arise from these studies. However, the TT-P haplotype of the σ_1 gene seemed to impact the vulnerability to AD. However, it did so differentially in different ethnic populations and its interaction with APOE ε4 allele remains to be understood.

15.4.2 Expression of σ_1 Receptors in AD

The impact of AD pathology on σ_1 receptor expression is still poorly documented, particularly as concerns precise densities in brain structures depending on the different phases of the disease. Using autoradiography and the non-selective σ ligand ^{3}H–DTG, a significant 26 % loss of binding sites was noted in the CA1 *stratum pyramidale* region of the hippocampus of AD patients as compared to healthy controls. This loss of σ receptors correlated with a 29 % loss of pyramidal cells [95]. Then, a loss of σ_1 receptors was observed with PET in the brain of patients with AD [90]. The binding potency of ^{11}C–SA4503 was significantly reduced in the frontal, temporal, and occipital lobe, cerebellum and thalamus of early AD patients as compared to healthy controls, but not in the hippocampus [90]. It may therefore be possi-

ble, that contrary to normal ageing, where decreases in neurotransmitter receptors expression could be related to a decrease in physiological plasticity that did not affect the σ_1 receptor, in AD, a loss of σ_1 receptor is observed and related to specific cell loss in vulnerable cellular population and brain structures.

15.4.3 Symptomatic Effects of σ_1 Receptors Ligands in AD

Since σ_1 receptor agonists are potent and wide-range anti-amnesic drugs, they could alleviate the memory deficits induced by amyloid toxicity in mouse and rat models of AD. It was initially reported that igmesine and PRE-084, in the low mg/kg dose-range, improved learning ability in the senescence-accelerated mouse SAMP/8 [56]. Then, (+)-pentazocine, PRE-084 and cutamesine, together with the σ_1 receptor-acting neuroactive steroids PREG, DHEA and their sulfate esters, PREGS and DHEAS, were tested in $A\beta_{25-35}$-injected mice. The toxicity was induced by a direct intracerebroventricular injection of oligomerized $A\beta_{25-35}$ peptide and after 1 week, when the brain toxicity closely relates to the toxicity observed in transgenic AD mouse models, animals were injected systemically with the σ_1 receptor ligands or the neuroactive steroids, several minutes before being tested in a series of behavioral tests [42]. All the σ_1 receptor agonists alleviated the $A\beta_{25-35}$-induced learning impairments and the effects were blocked by the σ_1 receptor antagonists haloperidol, BMY-14,802 or the neuroactive steroid progesterone, known to behave as a σ_1 receptor antagonist. These results were later confirmed, notably by Wang et al. [26] and Villard et al. [96, 97] who described the anti-amnesic effects of dimemorfan and ANAVEX1–41 or ANAVEX2–73, respectively, in the same mouse model. The compounds therefore appeared to be interesting symptomatic drugs in AD, with active doses similar or much lower than the references drugs donepezil, rivastigmine, galantamine or memantine [44]. It must be noted that no study addressed yet their symptomatic efficacy in a transgenic mouse model of AD.

15.4.4 Neuroprotective Effects of σ_1 Receptors Ligands in AD

The Holy Grail in AD research is a curative treatment that could, at the same time, block Aβ species generation (leading ultimately to the formation of senile plaques), prevent the hyperphosphorylation of Tau (responsible for the intracellular accumulation of neurofibrillary tangles), preserve mitochondrial integrity, boost neurite sprouting and dendrite connectivity and stimulate neurogenesis to repopulate neuronal cells circuitry. Presently available medications, the cholinesterase inhibitors donepezil, rivastigmine and galantamine or the NMDA antagonist memantine, are now understood as pure symptomatic drugs. A first step to unlock the discovery of an effective treatment in AD, would therefore be to define active neuroprotective drugs. Depending on their impact on Aβ load and Tau hyperphosphorylation, they could already help to preserve brain structure integrity and restore altered clearance systems for aggregated amyloid and Tau species. Since activation of the σ_1 receptor results in the brain in the modulation of numerous cytoprotective pathways, as we will detail below, σ_1 receptor agonists have been tested as putative neuroprotective drugs in AD. The in vitro evidence that PRE-084 or (−)-MR22 prevented $A\beta_{25-35}$-induced toxicity in rat neuronal cultures [98] was followed by the demonstration that in vivo, the σ_1 receptor agonists PRE-084, (−)-MR22, ANAVEX1–41, ANAVEX2–73 and PREGS had a neuroprotective action in pharmacological models of AD [44, 96, 97, 99, 100]. The compounds were injected either once before the $A\beta_{25-35}$ peptide or repeatedly o.d. during 1 week after the peptide injection. The Aβ peptides were injected either intracerebroventricularly or locally into the hippocampal formation and alone or associated with 192 IgG-saporin to induce a more severe cholinergic lesion [99]. Prevention of the learning deficits were evidenced. These beneficial effects

were accompanied by a general protection against the toxicity induced by amyloid peptides injection. Markers of oxidative stress, neuroinflammation, induction of apoptotic pathways, and cell loss were also attenuated by the repeated injection of σ_1 receptor agonists [44, 96, 97, 99, 100]. Interestingly, two studies suggested that selective σ_1 receptor agonists, namely (−)-MR22 in Antonini et al. [99] and PRE-084 in Lahmy et al. [101], could decrease amyloid load, since diminution of the increases in APP and $A\beta_{1-42}$ contents induced in the mouse brains after 192 IgG-saporin and/or $A\beta_{25-35}$ injection were prevented by the σ_1 receptor ligands. Lahmy et al. [101] also examined the activation of the main kinase involved in Tau protein hyperphosphorylation, namely the glycogen synthase kinase 3β (GSK-3β), and the level of hyperphosphorylated Tau at physiological or pathological epitopes in $A\beta_{25-35}$-treated mice. They reported that PRE-084 or ANAVEX2–73 decreased GSK-3β activation and Tau hyperphosphorylation. These two observations are particularly important since they coherently suggested that a chronic treatment with a σ_1 receptor agonist can not only allow a symptomatic improvement of memory ability for AD patients or a marked neuroprotection, but also have an effective impact on the accumulation of amyloid species and hyperphosphorylated Tau. This suggested that σ_1 receptor agonists could be altogether symptomatic, neuroprotective and disease-modifying drugs. Extensive studies in transgenic models must be undertaken to confirm this promising observations. A first result was published recently by Fisher et al. [102] based on AF710B, a compound acting partly as a σ_1 receptor agonist. The compound, administered at 10 μg/kg during 2 months, in female 3xTg-AD mice, attenuated the learning impairments in the water-maze, and decreased BACE1 level, GSK3β activity, p25/CDK5 level, or neuroinflammation. Interestingly, AF710B also attenuated soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ accumulations, the number of plaques and Tau hyperphosphorylation, confirming that such compounds, acting as σ_1 receptor agonist and M1 mACh receptor agonist (orthosteric, allosteric or bitopic) could be neuroprotective and putatively disease-modifying by improving amyloid and Tau pathologies [102].

15.4.5 The Involvement of S1R in AD Etiology

Exogenous administration of σ_1 receptor agonist alleviated the amyloid toxicity in acute as well as chronic AD mouse models. The question therefore appears to be whether an endogenous σ_1 receptor activity might be induced following a neurodegenerative insult, suggesting that the σ_1 receptor could be an endogenous neuroprotection system. The question was addressed recently in a series of studies combining invalidation of the σ_1 receptor expression (using σ_1 KO mice or repeated NE-100 treatment) and induction of the amyloid toxicity (using $A\beta_{25-35}$ injection or cross-breeding with APP_{Swe} mice to generate APP_{Swe}/σ_1 KO mice) [103–105]. Interestingly, the studies led to different results. When homozygous σ_1 KO mice received $A\beta_{25-35}$ at 1, 3 or 9 nmol intracerebrally 7 days before analyses of memory deficits using spontaneous alternation or passive avoidance and then analyses of the level of lipid peroxidation in the hippocampus, all three doses led to behavioral deficits of higher intensity in σ_1 KO mice and the two highest doses to increased lipid peroxidation levels [103]. In wild-type animals, only the 9 nmol dose is effective. A repeated NE-100 treatment from day −1 to 4 after the peptide resulted in a facilitation of the toxicity, significant at the 3 nmol dose [103]. Moreover in a recent study, the σ_1 receptor expression was invalidated in APP_{Swe} mice, and memory ability, level of oxidative stress, synaptic markers, plasticity genes and Aβ load analyzed at different time points for each gender and strain [104]. APP_{Swe}/σ_1 KO mice showed significantly decreased survival as compared with APP_{Swe}/Wt, Wt/σ_1 KO or even Wt/Wt controls. The spontaneous alternation response of APP_{Swe}/σ_1 KO animals was lower than single transgenic and control lines between 2 to 12 months of age. 8-m.o. APP_{Swe}/σ_1 KO mice showed impaired place learning in the water-maze and increased

ROS level in the hippocampus, but expression of hippocampal synaptic markers (PSD95, synaptophysin) was unchanged [104]. It therefore appeared from these experiments that invalidation in σ_1 receptor expression worsened Aβ toxicity and behavioral deficits in transgenic mice.

However, Yin et al. [105] injected $A\beta_{25-35}$ in heterozygous σ_1 KO mice and observed that the peptide injection impaired spatial memory with approximately 25 % death of pyramidal cells in the hippocampal CA1 region of wild-type mice, whereas it did not cause such impairments in heterozygous σ_1 KO mice. The $A\beta_{25-35}$ injection in wild-type mice increased levels of NMDA-activated currents and NR2B phosphorylation in the hippocampal CA1 region after 48 h followed by a 40 % decline at 72 h, which was inhibited by NE-100 [105]. However, the $A\beta_{25-35}$ injection in heterozygous σ_1 KO mice induced a slight increase in the NMDA-activated currents and NR2B phosphorylation at 48 h or 72 h as compared to non-injected heterozygous σ_1 KO mice. Treatment with PRE084 caused the same changes in NMDA-activated currents and NR2B phosphorylation as those in $A\beta_{25-35}$-treated wild-type or heterozygous σ_1 KO mice. These results suggested that the σ_1 receptor partial deficiency can reduce $A\beta_{25-35}$-induced neuronal cell death and cognitive deficits through suppressing $A\beta_{25-35}$-enhanced NR2B phosphorylation. They are not completely contradictory with the data from Maurice et al. [103, 104] since they are not involving a complete inactivation of σ_1 receptor, but bring an unexpected observation on the impact of σ_1 receptor in amyloid toxicity that remains to be clarified.

15.5 Cellular Mechanisms of the σ_1 Receptor-Mediated Neuroprotective Activity

15.5.1 Oxidative Stress, Mitochondrial Integrity and the σ_1 Receptor

There is extensive literature supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of AD. Oxidative damage precedes the onset of significant plaque pathology [106]. Aβ species target mitochondria [107] and gradual mitochondrial dysfunction in turn causes progressive neuronal dysfunction by different pathways including energy deprivation, oxidative stress, and cytochrome C release. Ultimately, necrosis and apoptosis is induced by Aβ accumulation. Altered mitochondrial functionality was reported in in vitro assays of direct application of Aβ peptides on isolated mitochondria as well as in vivo in $A\beta_{25-35}$ injected mice [108, 109]. In general, decline of mitochondrial functionality is demonstrated by reduced respiration rates, decreased complex IV activity and increased oxidative stress and proapoptotic markers. A direct interaction between Aβ and complex IV enzymatic subunits could be one the possible mechanisms for the disruption of complex IV activity, and complex I is also seen to be the target of Aβ peptide [110, 111]. The σ_1 receptor is mainly located at MAM and the outer mitochondrial membranes. It can be activated by ROS to modulate Bcl-2 expression [14] or its activation promotes a mild ROS response and its association with Rac1-GTPase in physiological conditions [16]. Considering that the mitochondrial impact of σ_1 receptors may drive its neuroprotective activity, the possible involvement of the chaperone protein in the defensive mechanisms against Aβ toxicity was studied and demonstrated using different σ_1 receptor ligands. Lahmy et al. [109] showed that in $A\beta_{25-35}$ injected mice, PRE-084 and ANAVEX2–73 both managed to reverse state 3 and uncoupled respiration deficits. How σ_1 receptor stimulation manages to increase respiration rates of altered mitochondria is still unclear. Several hypotheses could be proposed via controlling ROS levels, stabilizing mitochondrial membrane potential or mitochondrial respiratory chain complexes, or physically guarding and protecting the entrance of Aβ peptide inside mitochondria. Moreover, as Aβ accumulation in the brain is one of the neuropathological hallmark of AD, several studies have been conducted to find the physical appearance of Aβ in mitochondria [107]. It appeared that the translocase of the outer mem-

brane (TOM) complex is a major importer of Aβ into mitochondria [112]. Immunoelectron microscopy studies on human brain biopsies of mitochondrial fractions and assessing in vitro Aβ import showed a consistent localization pattern of Aβ to the mitochondrial cristae. Preincubated mitochondria with antibodies directed towards TOM20, TOM40, or TOM70 and then performed import assays showed a decreased import of $A\beta_{1-40}$ and $A\beta_{1-42}$ [112]. The fact that TOM complex with respect to their activity and signaling is in the close proximity to MAM, and therefore to the σ_1 receptor, may suggest a possible direct interaction. Finally, Smilansky et al. [113] recently showed that voltage-dependent anion channel 1 (VDAC1) and VDAC1 N-terminal peptide are involved in cell penetration and cell death induction by Aβ peptide [113]. VDAC1 is physically linked to the type-1 IP_3 receptor through the molecular chaperone GRP75, the cytosolic GRP75 connects the ligand-binding domain of the IP_3 receptors to VDAC1 [114] and σ_1 receptor indeed is a chaperoning protein for IP_3 receptor [77]. It appears therefore that the mitochondrial, and more precisely MAM, localization of σ_1 receptors as well as its chaperone activity [1] and its sensitivity to oxidative stress [14] is likely to be the main site of action for its neuroprotective activity, particularly in AD.

15.5.2 σ_1 Receptor Involvement in NGF-Mediated Trophic Effects

The σ_1 receptors not only facilitate BDNF release and effects, as detailed previously, but their activity may also be related to some NGF-mediated effects. NGF acts as a neurotrophic factor for cholinergic neurons of the basal forebrain. It is is present in the target areas of cholinergic neurons and affects their survival, fiber growth, and expression of transmitter-specific enzymes [115]. NGF prevents the degeneration of cholinergic neurons in adult rats with experimental cholinergic lesions and in AD models. Moreover, NGF stimulates neurite sprouting in models of restoration from lesion as well as a consequence of the action of several antidepressants. Takebayashi et al. [116] showed that σ_1 receptors might participate in the neurite sprouting in vitro in PC12 cells. (+)-Pentazocine, as well as the σ_1 active antidepressants imipramine and fluvoxamine, although ineffective by themselves, were found to enhance the NGF-induced neurite sprouting in PC12 cells in a dose-dependent manner [116]. The overexpression of σ_1 receptor in PC12 cells enhanced per se the NGF-induced neurite sprouting, while administration of σ_1 targeting antisense deoxyoligonucleotides attenuated it. This was later confirmed for fluvoxamine, but not sertraline or paroxetine, and for cutamesine, PPBP, DHEAS, donepezil, ifenprodil or novel σ_1-acting arylalkenylamines [117–121]. The potentiation of NGF-induced neurite outgrowth by selective σ_1 receptor agonists involved a subsequent interaction with IP_3 receptors, PLC-γ, PI_3K, p38MAPK, JNK, and the Ras/Raf/MAPK signaling pathways. Interestingly, this response appears to be highly pertinent to identify new antidepressants interacting with the σ_1 receptor. Ishima et al. [122] recently examined the effects of the selective serotonin reuptake inhibitors fluvoxamine, paroxetine, sertraline, citalopram and escitalopram, the serotonin and noradrenaline reuptake inhibitors duloxetine, venlafaxine and milnacipran, and the noradrenaline and specific serotonergic antidepressant mirtazapine, on NGF-induced neurite outgrowth in PC12 cells. Fluvoxamine, fluoxetine, escitalopram, and mirtazapine significantly potentiated NGF-induced neurite outgrowth in the cell assay. The effects of all drugs but not mirtazapine were antagonized by NE-100. Interestingly, the effects of fluvoxamine and fluoxetine were also antagonized by sertraline, indicating that the antidepressant may be a σ_1 receptor antagonist. These data therefore showed that most of the effects of antidepressants on NGF-induced neurite outgrowth involved an activation of the σ_1 receptor. Among the compounds tested, only mirtazapine independently potentiated neurite outgrowth in PC12 cells [122]. These results confirmed the importance of the σ_1 receptor

activity in some trophic responses to NGF. This NGF/σ_1 receptor interaction was mainly examined with antidepressants but could be extended to the cognitive actions of σ_1-acting neuroprotective drugs.

15.5.3 An α_7 nACh/σ_1 Receptor Interaction in Neuroprotection?

A putatively interesting target of σ_1 receptor activity, if not a direct partner, could be the α_7 nACh receptor. Indeed, several observations relate the effects of σ_1 receptor ligands to a rapid activation of α_7 nACh receptors. First, Yang et al. [100] analyzed the neuroprotective effect of PREGS in $A\beta_{25–35}$-injected mice. They observed that the effect of PREGS against $A\beta_{25–35}$-induced hippocampal pyramidal cells loss and PI3K-Akt activation or ERK inhibition were blocked by a σ_1 receptor antagonist or an α_7 nACh receptor antagonist. A σ_1 receptor agonist or a α_7 nACh receptor agonist mimicked the neuroprotective effect of PREGS on $A\beta_{25–35}$-induced pyramidal cell loss [100]. They concluded that, besides the known direct activation of each target by PREGS [67, 123], the neurosteroid-induced activation of the σ_1 receptor further modulates the function of α_7 nACh receptors [100]. More recently, Maurice [124] described in the same AD mouse model, a synergistic protective effect between donepezil and the σ_1 receptor agonist PRE-084 or ANAVEX2–73 against $A\beta_{25–35}$-induced learning deficits in the spontaneous alternation and passive avoidance tests. Using selective antagonists, it was shown that the (PRE-084 + donepezil) combination involved activation of both α_7 nACh receptors and σ_1 receptor, but not $\sigma_4\beta_2$ nACh receptor nor mACh receptors, and that the two targets act synergistically [124].

Stimulation of α_7 nACh receptors by agonists or positive allosteric modulators (PAMs) is an effective strategy to enhance cognition, particularly in AD [125]. Numerous compounds are in development and, in preclinical models, they not only are potent anti-amnesic drugs but also protect against Aβ toxicity and excess glutamate-induced toxicity. Aβ binds α_7 nACh receptors where it exerts a biphasic effect with inhibition at high doses [126]. Moreover, α_7 nACh receptors are expressed in mitochondria outer membrane, where they regulate the voltage-dependent anion channel-mediated Ca^{2+} transport and mitochondrial permeability transition [127]. A direct interplay between σ_1 receptors and α_7 nACh receptors is therefore conceivable. The α_7 nAChR is a Ca^{2+} ionophore that is allosterically modulated by Ca^{2+} [128]. It thus can be directly impacted by Ca^{2+} modulators, as is the σ_1 protein [77]. Second, the α_7 nAChR desensitizes rapidly. This has been a major concern in the development of α_7 nAChR agonists as putative drugs. The σ_1 receptor acting drugs could directly impact the desensitization kinetics and bioavailability of α_7 nAChR by chaperoning several components of the plasma membrane, including support proteins and membrane receptors, and thus modulate plasma membrane composition [129–132]. These mechanisms and the close localization of σ_1 receptor and α_7 nAChR at the mitochondrial membrane favor a close interplay of the two receptors in mediating cytoprotection.

15.6 Ligand Specificity – Innovative Tracks to Design New σ_1 Receptor-Acting Ligands in Learning and Memory

15.6.1 Selective Vs. Non-Selective σ_1 Ligands

Drug development programs are currently very active in the field of cognitive or cytoprotective activities of σ_1 receptor ligands. A plethora of molecular profiles are tested and several interesting features have arisen. A first pertinent question arising from the pharmacological profiles of the lately described compounds concerns the pharmacological mode of action of the compounds at the σ_1 receptor. One aspects is that σ_1 receptor drugs are rarely highly selective. Due to the poor constraint of the σ_1 receptor pharmacophore described historically [133, 134],

plethora of compounds acting primarily at other targets, also bind the σ_1 receptor and sometimes with very high affinity. Some non-selective compounds are in development but some others are already in clinical use and recent data confirms that they are acting partly through their σ_1 receptor activity, among them donepezil and fluvoxamine. Donepezil is a cholinesterase inhibitor that is very selective for acetylcholinesterase as compared with butyrylcholinesterase [135], but which also presents a very low nanomolar affinity for the σ_1 receptor [136]. The compound is anti-amnesic in pharmacological and pathological models of amnesia in rodents and humans (for reviews, see [137–139]). The drug prevented dizocilpine-induced learning impairment in mice and the effect was blocked by the σ_1 receptor antagonist BD1047 or pretreatment of the animals with a σ_1 receptor-targeting antisense oligodeoxynucleotide treatment [140]. A subchronic 14-days treatment with donepezil alleviated the PCP-induced cognitive deficits in a novel object recognition test, in a NE-100-sensitive manner, showing the σ_1 receptor involvement in the symptomatic effects of the drug [46]. The compound also shows some neuroprotective activity in in vitro and in vivo models of amyloid toxicity [44, 141, 142] or in transgenic animals [143, 144]. We previously described that, in the $A\beta_{25-35}$ mouse model of AD, when donepezil was injected o.d. after the amyloid peptide, it prevented the appearance of learning deficits and oxidative stress with an effect partly sensitive to a pre-administration of the σ_1 receptor antagonist BD1047 [44]. Other cholinesterase inhibitors tacrine, rivastigmine and even galantamine, were effective using the same administration protocol. When donepezil was administered only once, just before the amyloid peptide, the drug still prevented the appearance of learning deficits and oxidative stress. Interestingly, the effects were completely blocked by BD1047 and not shared by the other cholinesterase inhibitors [44]. The observations that neuroplastic effect of donepezil, like the NGF-induced neurite outgrowth in PC12 cells, has been shown to be blocked by co-administration of NE-100 or the IP_3 receptor antagonist xestospongin C [118], and that donepezil, with an ED_{50} of 1.29 mg/kg, was found to dose-dependently occupy a significant fraction of the σ_1 receptor population in the rat brain [145] confirmed that a σ_1 receptor agonist component is involved in the pharmacological action of the drug.

Fluvoxamine inhibits serotonin reuptake with an K_i of 3 nM [146] and binds to the σ_1 receptor with an IC_{50} of 17–36 nM [147, 148]. The antidepressant has been shown to activate the σ_1 receptor in a variety of cellular and behavioral responses, including the potentiation of nerve growth factor-induced neurite outgrowth in PC12 cells [116, 117], the enhancement of BDNF-induced glutamate release via the PLC-γ/IP_3/Ca^{2+} pathway [88], or the prevention of ER stress in neuroblastoma cells by increasing σ_1 receptor expression and ATF4 translation directly, through the σ_1 receptor activation, without involvement of the PERK pathway [149]. Moreover, similarly as observed with donepezil, a subchronic treatment during 2 weeks with fluvoxamine alleviated in a NE-100 sensitive manner, the cognitive deficits induced in mice by phencyclidine [45] and that, using ^{11}C–SA4503 as a dynamic PET radiotracer, fluvoxamine was shown to bind to σ_1 receptor in all brain regions in living human brain in a dose-dependent manner [150]. Subsequently, fluvoxamine has been proposed to improve cognitive impairments in patients with schizophrenia through its σ_1 receptor agonist activity [151].

In the development of neuroprotective drugs in AD, the mixed mACh receptor/σ_1 receptor agonists belonging to the tetrahydroaminofurane family, ANAVEX1–41 and ANAVEX2–73, have been shown to be very promising compounds. A clear synergy exists between the different targets of the molecules, M1 mACh receptor agonism and σ_1 receptor agonism for ANAVEX1–41 and likely M2 mACh receptor antagonism and σ_1 receptor agonism for ANAVEX2–73 ([150], unpublished data). The result is that the first compound is active at extremely low (µg/kg) doses in vivo in rodents, either regarding its anti-amnesic or cytoprotective effects [96, 152, 153]. The second is active in vivo in the same dose-

range as donepezil (low mg/kg dose in rodents) although it has in vitro affinities in the high micromolar concentration range [97]. A bicyclic heterocyclic spiro compound, AF710B, was recently described also having a good neuroprotective efficacy in mouse models of AD with a similar pharmacological profile as allosteric M1 mACh receptor and σ_1 receptor agonist [102]. The mechanism of the synergy has still to be established but several hypotheses have been proposed. First, the σ_1 receptor activity could increase, in the cortex and hippocampus, the glutamatergic transmission and long-term potentiation, since σ_1 receptor activation facilitates glutamate release and activates glutamate receptors [66, 154]. The drug could also increase ACh release, via pre-synaptic σ_1 receptor-mediated and M_2 mACh autoreceptor-mediated effects [40, 75]. The σ_1 receptor activity can amplify the mACh receptor-induced PLC activity [155, 156] and induce IP_3 formation and activation of ER IP_3 receptors [77, 157]. These transduction mechanisms sustain learning and memory processes and are markedly affected by amyloid peptides. Alternatively, Fisher et al. [102] proposed that σ_1 receptor could heterodimerize with M1 mACh receptors and the mixed drugs could directly address the M1 mACh receptor/σ_1 receptor heterodimers, which existence, signalization and ligand binding dynamics remain to be determined.

15.6.2 σ_1 Receptor Positive Allosteric Modulators (σ_1 PAMs)

A second recent development that could also be very promising concerns several compounds that have been described as PAMs of the σ_1 receptor. First, the benzazepine derivative SKF83959 dose-dependently increased the binding of the prototypic σ_1 receptor radioligand ^{3}H(+)-pentazocine in the rat brain and liver tissues by an allosteric mechanism, as shown by saturation and kinetic experiments [158]. The compound suppressed the expression or release of the pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), and inhibited the generation of reactive oxygen species in lipopolysaccharide (LPS)-stimulated BV2 microglia [159]. All of these responses were blocked by selective σ_1 receptor antagonists BD1047 or BD1063. The drug also promoted the binding activity of DHEA to σ_1 receptor and enhanced the inhibitory effects of DHEA on LPS-induced microglia activation in a synergic manner (1597). Second, Zvejniece et al. [30] described E1R, a phenylpyrrolidin acetamide derivative, which failed to affect ^{3}H(+)-pentazocine binding but enhanced PRE-084-induced stimulating effect in the electrically stimulated rat vas deferens assay and bradykinin-induced $[Ca^{2+}]_i$ increase assay. Pretreatment with E1R facilitated passive avoidance retention in a dose-related manner. Furthermore, E1R alleviated the scopolamine-induced cognitive impairment in the passive avoidance and spontaneous alternation tests in mice [30]. The in vivo and in vitro effects of E1R were blocked by treatment with NE-100. Such PAMs are currently developed for several targets, like α_7 nACh receptor PAM in memory, neuroprotection and schizophrenia. They bring the advantage of boosting the physiological response without inducing a direct response by themselves and therefore appear largely devoid of side-effects. The preliminary observations that σ_1 receptor PAMs could be as effective as direct σ_1 receptor agonists on learning and memory responses is particularly promising since, taken into account the low level of side-effects associated with σ_1 receptor activation, PAMs could be extremely safe and active drugs, suitable candidates for combination therapy strategies in numerous indications.

15.7 Conclusions

As reviewed, pre-clinical evidence for a role of σ_1 receptors in learning and memory processes and neuroprotection has accumulated. The availability of numerous compounds from different chemical families with high affinity, high selectivity, or shared selectivity with pertinent targets, and very good bio-availability suggests that σ_1-acting

therapeutic drugs could be developed successfully. The mechanism of action of σ_1 receptors is currently subject to extensive research and compilation of historical data and recent advances allows to propose a global scheme. However, the more we progress in characterizing the σ_1 receptor nature and cellular actions, the more pleiotropic it appears. The σ_1 receptor indeed modulate neurotransmitter systems directly, through release and signaling systems, and indirectly through Ca^{2+} mediated signaling and impact on other regulators as trophic factors, cytokines and mediators, or physiological effects on oxidative stress, transcription factors and gene regulation. σ_1 Receptors induce both short-term effects and long-term plasticity changes in nerve cells, affects several types of cells and may also induce different effects depending on agonist concentration and ligand interaction with different biophysical forms as monomer, dimer, tetramer…. This complexity, even increased by evident differences between physiological and pathological conditions, is at the same time a challenge and an opportunity for drug development. No doubt the clinical trials presently in progress will help to validate the clinical interest of the σ_1 receptor as a therapeutic target.

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16 Sigma-1 Receptor in Motoneuron Disease

Renzo Mancuso and Xavier Navarro

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease affecting spinal cord and brain motoneurons, leading to paralysis and early death. Multiple etiopathogenic mechanisms appear to contribute in the development of ALS, including glutamate excitotoxicity, oxidative stress, protein misfolding, mitochondrial defects, impaired axonal transport, inflammation and glial cell alterations. The Sigma-1 receptor is highly expressed in motoneurons of the spinal cord, particularly enriched in the endoplasmic reticulum (ER) at postsynaptic cisternae of cholinergic C-terminals. Several evidences point to participation of Sigma-1R alterations in motoneuron degeneration. Thus, mutations of the transmembrane domain of the Sigma-1R have been described in familial ALS cases. Interestingly, Sigma-1R KO mice display muscle weakness and motoneuron loss. On the other hand, Sigma-1R agonists promote neuroprotection and neurite elongation through activation of protein kinase C on motoneurons in vitro and in vivo after ventral root avulsion. Remarkably, treatment of SOD1 mice, the most usual animal model of ALS, with Sigma-1R agonists resulted in significantly enhanced motoneuron function and preservation, and increased animal survival. Sigma-1R activation also reduced microglial reactivity and increased the glial expression of neurotrophic factors. Two main interconnected mechanisms seem to underlie the effects of Sigma-1R manipulation on motoneurons: modulation of neuronal excitability and regulation of calcium homeostasis. In addition, Sigma-1R also contributes to regulating protein degradation, and reducing oxidative stress. Therefore, the multi-functional nature of the Sigma-1R represents an attractive target for treating aspects of ALS and other motoneuron diseases.

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Sigma-1 receptor • Motoneurons • Motoneuron disease • Amyotrophic lateral sclerosis • Etiopathology

16.1 Introduction: Motoneuron Diseases

Motoneuron diseases (MND) are progressive neurodegenerative disorders of wide etiology and clinical spectra, but with a common feature: the loss of lower and/or upper motoneurons. Amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) are the most frequent forms of MND and therefore the most studied. ALS was first described by Charcot in 1869 and is the most common type of MND in adults, with an incidence of 1–5 per 100.000. Concomitant degeneration of both upper (corticospinal/corticobulbar) and lower (spinal/bulbar) motoneurons distinguishes ALS from other forms of MND [1–3]. The main neuropathological features of ALS include extensive loss of motoneurons in the anterior horns of the spinal cord and motor nuclei of the brainstem, degeneration of the corticospinal tract, and degeneration and loss of large pyramidal neurons in the primary motor cortex, also accompanied by reactive gliosis around the areas of degeneration [3]. Cytoplasmic protein inclusions are common in the degenerating neurons, which predominantly comprise a nuclear RNA processing protein, TDP-43 (TAR-DNA binding protein 43) [4] It has been classically considered that despite most ALS cases are sporadic (sALS), 5–10 % are familiar (fALS), related with several genetic mutations [1, 5]. No matter if they are sporadic or familiar, patients develop progressive weakness and muscle atrophy, with spasticity and contractures. Progressive weakness may start distally or proximally in the upper or lower limbs and finally affect all muscles, including those related with breathing, speaking and swallowing. Patients die, mostly due to respiratory failure, by 2–5 years after diagnosis [2, 6].

No effective treatment is presently available for ALS [1]. Patient care focuses on symptomatic treatments and physical therapy. Assisted ventilation and nutrition can transiently overcome the loss of upper airway and respiratory muscular control [2]. A large number of therapeutic trials have been attempted, but it was not until the early 1990s that the first drug approved by the FDA for the treatment of patients with ALS reached the market: riluzole, an antiglutamatergic agent that blocks the presynaptic release of glutamate. However, the efficacy of riluzole is questionable, with minimal therapeutic benefits of about 3–4 months of survival increase [7]. One of the main concerns for developing new therapies is the lack of direct translation from promising preclinical findings to successful clinical results. Although the heterogeneous and complex nature of ALS has been studied extensively, the absence of early detection markers and proper biomarkers for the disease evaluation of patients does not allow identifying whether patients are at different stages or even developing the disease because of different underlying causes. These drawbacks often lead to a difficult interpretation of the results from clinical studies. In this sense, patients who develop the disease mainly because of defects in a particular pathway would display greatest benefit from the compounds that selectively target that pathway. Interestingly and supporting this idea, in most clinical trials, a subset of subjects showed improvement, but none of the compounds displayed an overarching effect on most patients. Therefore it seems that each clinical trial has been successful only in a select subset of individuals. Since ALS is a multifactorial disease, future strategies should be focused on multi-target drugs or on combinatorial treatments that might maximize the translational effects [1].

Frontotemporal lobal degeneration (FTLD or FTD) is the second most common type of dementia after Alzheimer's disease. It is caused by progressive neuronal atrophy and loss in the

frontotemporal cortex, and is characterized by personality and behavioral changes, as well as gradual impairment of language skills [8]. Traditionally, ALS and FTLD were considered as two distinct identities. However, novel evidence suggests that both pathologies belong to a clinical continuum, with pure forms linked by overlapping syndromes. The first link established between FTLD and ALS was the identification of TDP-43 positive ubiquitinated cytoplasmic inclusions in almost all ALS and more than a half FTLD patients [8, 9]. Although neuropsychological testing shows normal cognition in the majority of ALS patients, up to 50 % of them may present some degree of cognitive impairment, while 15–18 % meet the criteria for FTLD [10]. On the contrary, few patients with FLTD develop ALS [11]. Indeed, FTLD-only, ALS-only and coincident FTD-ALS cases were reported to occur inside the same family, supporting the hypothesis of a link between both pathologies. The recent finding of an hexanucleotid expansion in C9ORF72 constitutes a strong link between ALS and FTLD [12–15].

16.2 Pathophysiological Mechanisms Underlying Motoneuron Death

The exact molecular pathway causing motoneuron degeneration in ALS is unknown, but as with other neurodegenerative diseases, it is likely to be a complex interplay between multiple pathogenic mechanisms that may not be mutually exclusive and in which is still unknown the causative relation between them or whether they are the consequence of an upstream disturbance [1, 5, 16].

The identification of underlying genetic defects of familial cases of ALS has allowed the development of relevant animal models of the disease in mice, rats, zebra fish and drosophila [1, 4, 17–19], which have been essential for uncovering morphological and molecular pathogenic events in vivo that are not possible to investigate in humans. The most widely used ALS models are transgenic mice over-expressing human mutated forms of the SOD1 gene, which recapitulate the most relevant clinical and histopathological features of both familial and sporadic ALS.

Among the proposed pathophysiological mechanisms, excitotoxicity has been deeply explored. Neuronal injury caused by excitatory mediators may be due to failure in the neurotransmitter clearance from the synaptic cleft or increased postsynaptic sensitivity to glutamate. This enhanced excitatory input induces a massive calcium influx into the cytoplasm that damages the cells through the activation of calcium-dependent proteases, lipases and nucleases. A large body of evidence implicates excitotoxicity as a mechanism contributing to motoneuron death in ALS, such as threefold increased levels of glutamate in CSF from ALS patients [20, 21]. Furthermore, overactivation of NMDA receptors and increased calcium permeability of AMPA receptors have been described in ALS mouse models [1, 22–25]. Loss of the glial excitatory amino acid transporter 2 (EAAT2) was also reported in ALS mouse models [26, 27].

Oxidative stress results from the imbalance between the production of reactive oxygen species (ROS) and the biological capacity to remove ROS or repair ROS-induced damage. The analysis of CSF and serum from ALS patients showed increased concentration of ROS compared to healthy subjects [28–31]. Evidence of oxidative stress damage to proteins [32], lipids [30] and DNA [33] was also reported to occur in ALS patients. Oxidative stress has been also documented in ALS mouse models [34, 35].

Mitochondria are the cellular organelle in charge of ATP production, calcium homeostasis maintenance and intrinsic apoptosis regulation. An important core of evidences implicates mitochondria as key players in ALS physiopathology [36]. Reduced mitochondrial DNA content associated with increased mutations of mitochondrial DNA, and respiratory chain complexes dysfunction have been described in the spinal cord of ALS patients [37]. Mitochondrial function impairments affect also the skeletal muscle of ALS patients [38]. In vitro studies showed mitochondrial morphological and functional altera-

tions in NSC-34 cells expressing mutant SOD1 [39]. Experiments performed in mSOD1 mice also revealed early mitochondrial morphological abnormalities prior to onset of symptoms [40].

Neurons are polarized cells that require efficient mechanisms to direct axonal vs. dendritic transport. Since neurons transmit signals along long distances, proteins and organelles have to travel more than in other cell types (axons of human motoneurons can reach 1 m long). Even within the axon, cargos must be delivered to specific compartments, thereby increasing the importance of axonal transport in motoneurons. Several works demonstrated the accumulation of neurofilaments in motoneuron cell bodies in human patients, suggesting that axonal transport is impaired in these cells [41–44]. Additionally, abnormalities of organelle axonal trafficking occur in ALS patients [45]. Axonal transport has been widely studied in animal models mimicking ALS. It has been demonstrated that transgenic mice overexpressing SOD1 transgene develop neuronal cytoskeletal pathology resembling human ALS [46]. Controversially, recent evidence suggests that axonal transport deficits may evolve independently from motoneuron degeneration in mutant SOD1 mice [47]. Marinkovic et al. [47] demonstrated that mutant SOD1 axons are able to survive despite long-lasting transport deficits since these are present soon after birth, months before the first signs of muscle denervation [48–50].

Protein aggregates or inclusions have long been recognized as a pathological hallmark of several neurodegenerative disorders, including ALS, in which protein aggregates are common in spinal motoneurons [1]. Ubiquitin-positive inclusions are characteristic of ALS histopathology. Nevertheless, it remains unclear whether inclusion formation is responsible for cellular toxicity and ALS pathogenesis, if aggregates may be innocuous neurodegeneration-derived products, or if they may represent a protective reaction of the cell to reduce intracellular concentrations of toxic proteins. Several proteins are found forming the intracellular inclusions in ALS, including neurofilaments [42–44], SOD1 [51–53], TDP-43 [9, 54], FUS [55, 56], ubiquilin 2 [57] and C9OFF72 [12, 13].

Physiologically, accumulation of misfolded proteins elicits the endoplasmic reticulum (ER) stress response. ER-resident chaperones sense the accumulation of misfolded proteins and activate the Unfolded-Protein Response (UPR), which leads to the suppression of general translation and ER-associated protein degradation. However, prolonged UPR activation may trigger apoptotic signaling [58]. The addition of CSF from ALS patients induces ER stress on cultured NSC-34 cells and primary rat spinal motoneuron cells [59]. Considerable evidence implicates ER stress as an important feature of motoneuron degeneration in ALS. UPR markers are upregulated in sALS patients [60] as well as in mutant SOD1 rodent models [61, 62]. Interestingly, a longitudinal gene expression profile in mutant SOD1 mice revealed early upregulation of several UPR markers prior to muscle denervation in vulnerable motoneurons (innervating fast fatigable muscles, e.g. extensor digitorum longus) compared to resistant motoneurons (innervating slow muscle fibers, e.g. soleus). Similar changes eventually occurred in disease-resistant motoneurons but 25–30 days later [62], suggesting a role for ER stress in determining the susceptibility of motoneurons in ALS.

Neighboring glial cells also play a crucial role in the motoneuron degeneration occurring in ALS [1, 63, 64]. Clement et al. [65] generated chimeric mice expressing mSOD1 in specific cell lines and demonstrated that normal motoneurons developed ALS signs when surrounded by mutant SOD1-expressing glia. To further explore the contribution of microglia in ALS, double transgenic mice were generated expressing the Cre–Lox recombination system to selectively suppress the mutant SOD1 expression in motoneurons or microglia. Mutant SOD1 deletion in motoneurons lead to delayed disease onset but no modifications of disease progression once initiated. On the other hand, mutant SOD1 suppression in microglia and macrophages did not alter disease onset but significantly prolonged mice survival. These findings suggest that disease onset and

progression might be related to different mechanisms [66, 67]. It is also accepted that astrocytes play a role in ALS. Astrocytes derived from post-mortem tissue of familial and sporadic ALS patients are toxic to motoneurons but not to GABAergic neurons. Blocking mSOD1 expression produced significant neuroprotective effects on ALS-derived astrocytes [68].

Neuroinflammation is a common pathological event of neurodegenerative disorders [69] and its modulation has been proposed as an important potential therapeutic target [70]. In ALS, motoneuron damage leads to the activation of microglia, astrocytes and the complement system, further contributing to neurodegeneration [71, 72]. Spinal cord tissue and CSF from sporadic and familial ALS cases present increased microglial activation and T cells infiltration [73, 74] as well as higher concentration of some proinflammatory mediators, including monocyte chemoattractant protein 1 (MCP-1) and IL-8 [75]. Gene array analysis of mutant SOD1 mice revealed an enhanced expression of inflammatory-related molecules especially at late stages of the disease [76, 77].

RNA processing abnormalities were first related to MND by the description of Spinal Motor Neuron protein 1 (SMN1) mutations as a cause of SMA [78]. The SMN proteins play a role in the assembly of small ribonucleoproteins, which participate in pre-mRNA splicing [79]. Later identification of TDP-43, a RNA-DNA binding protein, as a major component of the ubiquitinated protein inclusions in ALS patients [9] focused the attention to RNA processing alteration as an important pathophysiological mechanism of the disease. TDP-43 is predominantly localized in the nucleus where it is implicated in several events for RNA processing, including transcriptional regulation, alternative splicing and microRNA processing. ALS-related TDP-43 positive cytoplasmic inclusions are present in neuronal and non-neuronal cells, excluding those based on mSOD1 and FUS mutations [54, 80]. Recent studies have evaluated the RNA-binding targets of TDP-43 [81–83] and revealed that TDP-43 binds to several RNA target molecules (about 30 % of the mouse transcriptome). Such high level of intronic binding suggests a nuclear function for TDP-43. In fact, blocking tardbp43 expression using antisense oligonucleotides in adult mouse striatum altered the expression levels of 601 mRNA and changed the splicing pattern of 965 mRNA transcripts, including some relevant to neurodegeneration, such as progranulin, choline acetyltransferase or FUS [81]. TDP-43 alteration might potentially alter the transcriptional process of crucial genes for motoneuron homeostasis. Additional evidence about dysregulated RNA processing as motoneuron injury contributor in ALS arises from the detection of RNA oxidation biomarkers in human ALS and mSOD1 mice [84]. Since the discovery of C9ORF72 hexanucleotide (G_4C_2) repeat expansions as a frequent cause of ALS and FTD, efforts have been conducted for investigating linked pathophysiological abnormalities. Repeat containing RNA foci in these patients suggested a deleterious gain of function. Repeats are able to form G-quadruplexes, which may be able to facilitate the binding and sequestration of different RNA binding proteins to the repeat [85]. Subsequently, these proteins are not able to execute their normal functions. Another mechanism is the possible occurrence of repeat-associated non-ATG (RAN) translation along the hairpin-forming repeat. This results in aggregates containing different dipeptide repeat proteins in patients with the C9ORF72 repeat expansion [15, 86]. Recently, two independent studies used engineered drosophila to express high repeat expansions of G_4C_2 [87, 88], and established a strong connection between defective nuclear trafficking and neurodegeneration in these flies.

16.3 Structure and Functions of Sigma-1 Receptor

The Sigma-1R is a transmembrane protein found in the ER [89, 90], which is highly expressed in motoneurons and other cells in the spinal cord [89, 91–94]. Although it was initially classified as an opioid receptor, further experiments showed that its properties were distinct from known opioid receptors [95]. A 223 amino acids Sigma-1R

protein has been cloned from several mammals, and contains 90 % identical and 95 % similar amino acid sequences across species, with both the N- and C-termini on the same side of the membrane facing the cytoplasm [90, 96], and can be present in monomeric or oligomeric forms even in the absence of ligand [97, 98]. The N-terminus, of approximately 110 amino acids, determines the diversity of intracellular interactions of Sigma-1R with a variety of proteins [99–102]. Many synthetic compounds have been characterized as selective modulators of the Sigma-1R [103], and several endogenous molecules have been proposed to be Sigma-1R ligands as well, including lipid steroids (DHEA, progesterone and pregnenolone sulfate) [104], lipid sphingosine derivatives [105] and N,N-dimethyl tryptamine (DMT) [106]. It is plausible that these compounds regulate Sigma-1R function in different tissues according to their availability.

This receptor has the ability to translocate from the ER to the plasma membrane and mitochondria-associated membranes [90, 107, 108]. In the nervous system, Sigma-1R mediates regulation of a wide range of processes, such as neuritogenesis [109], modulation of K+ channels [110] and N-methyl-D-aspartate (NMDA) receptors activity [111], ER-mitochondria communication [90], modulation of G-protein couples receptors (GPCRs), Ca2+ homeostasis [90], and microglial activity [112]). The Sigma-1R appears as a pluri-functional target involved in a broad range of cellular processes and, thereby, its modulation might provide better translational outcomes than drugs acting selectively on one of these multiple aspects.

Langa et al. [113] developed homozygous Sigma-1R knock out mice, which showed to be fully fertile and with no obvious behavioral alterations. However, further careful analyses revealed alterations of hippocampal neurogenesis [114, 115], ethanol consumption [116], retinal function [117, 118], anxiety, memory impairments [119] and, most relevant, motor dysfunction and loss of neuromuscular junctions [120, 121].

16.4 Sigma-1 Receptor and Motoneurons

To fully understand the mechanisms underlying Sigma-1R role in motoneurons, it is important to know its subcellular localization in the cells. It has been shown that Sigma-1R is enriched in the subsurface cisternae in postsynaptic C-terminals of motoneurons [120]. Synaptic innervation onto motoneurons is complex, with synapses involving all the major neurotransmitters, that have been classified as S, M, T F, P and C-boutons/terminals (referring to the pre- or postsynaptic structure, respectively) [122]. C-terminals are large cholinergic postsynaptic sites with a unique ultrastructure seen at the electron microscopy level. They are referred to as “C” because of the subsurface cisternae of smooth endoplasmic reticulum adjacent to the plasma membrane, and are large synapses found only on soma and proximal dendrites of motoneurons [123]. Presynaptic C-boutons originate from a group of cholinergic interneurons located near the spinal cord central canal, which have been shown to increase motoneuron excitability and, thus, potentiate muscle contraction [124]. Interestingly, Sigma-1R is specially enriched in the subsurface cisternae underlying the postsynaptic membrane of C-boutons in motoneurons. Diverse alterations of C-boutons have been reported in animal models of ALS and spinal cord injury [125–128]. The postsynaptic membrane of C-boutons is rich in numerous proteins, including m2-type muscarinic receptors (m2AChR) [129–131], voltage-gated Kv2.1 [132], Kv1.4, Kv1.5 [110] and Ca^{2+}-activated K^{+} (SK) channels [133], connexin 32 [134], VAMP-2 [129], Sigma-1R [135] and neuregulin-1 [136]; whereas the presynaptic element contains, at least, neuregulin-1 receptors ErbB2 and ErbB4 [136]. In contrast, the role of subsurface cisternae in postsynaptic densities where Sigma-1 receptors are located is still unknown, but believed to couple the electrical activity of the plasma membrane with intracellular signaling involving the ER [137, 138].

Cholinergic innervation onto motoneurons plays a role in modulating the excitability of the cells during locomotion [124, 139]. Interestingly, Sigma-1R, m2AChR and SK channels have special relevance regarding motoneurons excitability. Indeed, it has been proposed that differential expression of SK2.2 and SK2.3 channels in neurons is a marker for α-motoneurons innervating fast or slow muscle fibers modifying the hyperpolarization properties of the plasma membrane [133]. Miles et al. [139] described how cholinergic innervation on motoneurons increases excitability during fictive locomotion by acting on m2AChR, whereas motoneurons lacking Sigma-1R have increased excitability [140]. Sigma-1R has been also shown to interact with diverse potassium channels, thereby shaping neuronal excitability [99, 110, 141, 142] (Fig. 16.1).

Sigma-1R co-localizes with neuregulin-1 expressed at the motoneuron C-boutons postsynaptic membrane [136]. Neuregulin-1 is a neurotrophic factor essential for the normal development and function of the nervous system [143]. Neuregulin-1 ErbB receptors are also located in the presynaptic terminals of C-boutons. Neuregulin-1/ErbB system alterations have been related to ALS, with reduced neuregulin-1 type III expression in the spinal cord of ALS patients and mouse models [144]. Loss-of-function mutations on the gene encoding for ErbB4 receptor produce late-onset ALS in patients [145]. Although no link between Sigma-1R and neuregulin-1 has been established yet, it is likely that Sigma-1R serves as a chaperone for neuregulin-1 at subsurface cisternae of motoneurons, as it has been shown to participate in the post-translational processing of other neurotrophic factors [146] (Fig. 16.1).

Little is known about endogenous ligands for Sigma-1R. It has been shown that N,N-dimethyltryptamine (DMT) is an endogenous agonist for the Sigma-1R [147] and that Indole(ethyl)amine N-methyltransferase (INMT), the enzyme that converts the amino acid tryptophan into DMT, co-localizes with Sigma-1R at C-terminals of motoneurons [135]. Endogenous steroids have been shown to act as Sigma-1R agonists, including dehydroepiandrosterone (DHEA) sulfate and pregnenolone sulfate [148]. Nevertheless, further studies are needed to elucidate the mechanisms by which Sigma-1R function is endogenously modulated and how this affects motoneuron physiology (Fig. 16.1).

16.5 Evidences of Sigma-1 Receptor Contribution in Motoneuron Disease

There is a body of evidence suggesting that Sigma-1R alterations lead to motoneuron dysfunction and degeneration [121, 140]. Mutations in a highly conserved region of the transmembrane domain of the Sigma-1R were described in ALS patients. The mutation produces an aberrant subcellular distribution of the receptor in NSC34 cells, and cells expressing the mutant protein are more prone to undergo apoptosis induced by ER stress [149]. Sigma-1R was found to abnormally redistribute in alpha-motoneurons of ALS patients and form ubiquitinated aggregates that lead to UPR. Additionally, Sigma-1R levels were found reduced in samples of ALS patients [150]. Other mutations in the 3′-untranslated region (UTR) of the Sigma-1R gene were described in affected individuals with the FTD-ALS pedigree [151].

Interestingly, Sigma-1R KO mice display locomotor deficits associated with muscle weakness, axonal degeneration and motoneuron loss [121, 140]. Altered Sigma-1R function in motoneurons has been also shown to disrupt ER-mitochondria contacts and affect intracellular calcium signaling, leading to activation of ER stress and to defects in mitochondrial dynamics and transport [121]. Crossing Sigma-1R KO mice with mutant SOD1 mice (SOD1^{G93A}) exacerbated the motor phenotype and accelerated the end stage of the disease [140]. Conversely, stimulating Sigma-1R function using the agonists PRE-084 or SA4503 has been shown protective in both *in vitro* and *in vivo* models of mutant SOD1 ALS [94], as well as in non-SOD1 linked MND [152].

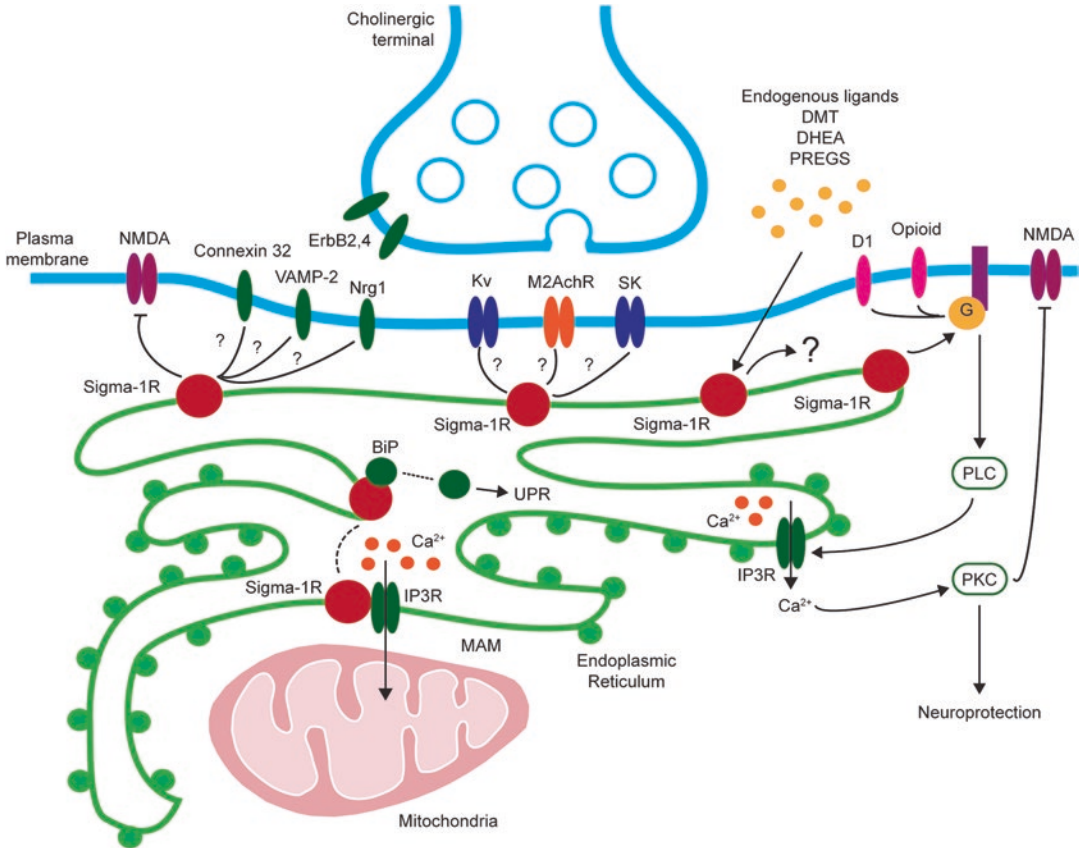

Fig. 16.1 Sigma-1 receptor localization at the C-boutons and its pleiotropic role in the motoneuron. Sigma-1 receptor is located at the endoplasmic reticulum subsynaptic cisterna of the cholinergic synapses, from where it may interact both with elements of the plasma membrane (e.g. ion channels) or the cytoplasm (e.g. mitochondria). Sigma-1 receptor modulates the activity of several ionotropic and metabotropic receptors, including M2AchR, NMDA, dopaminergic D1 and opioid receptors. Further studies are needed to elucidate how Sigma-1 receptor interacts with ion channels (Kv or SK) and other elements present at the C-boutons, such as Connexin32, VAMP-2 and Neuregulin1. The Sigma-1 receptor is also able to interact with BiP, a chaperone of the endoplasmic reticulum, and to participate in the interactions between the endoplasmic reticulum and the mitochondria. For further details, see the Sect. 16.4 text

A note of caution must be taken since there is controversy about the expression profile of Sigma-1R in mutant SOD1 ALS models. Analysis of Sigma-1R in protein extracts from lumbar anterior spinal cord showed no changes in the amount of Sigma-1R expressed [94], whereas immunohistochemical analysis revealed decreased labeling of Sigma-1R at the C-boutons of SOD1 lumbar motoneurons at early pre-symptomatic stages of the disease [127].

16.6 Potential Mechanisms on Sigma-1 Receptor-Mediated Therapeutic Actions

The Sigma-1R has been shown to be a target for the treatment of a variety of chronic neurological diseases, including pain [153–155], depression [148], Alzheimer's [156–158], Parkinson's [159], and Huntington [160] diseases, schizophrenia

[161], stroke [162, 163], ischemia [164], degeneration of retinal neurons [117, 118], and selective cholinergic lesions [156]. The administration of Sigma-1R ligands has promoted neuroprotection after several types of insults, including excitotoxic damage [165], hypoxia-mediated neurotoxicity [166], oxidative stress-induced cell death [167] and glucose deprivation [164].

Regarding motoneurons, the selective Sigma-1R agonist PRE-084 has been reported to exert positive effects on motoneuron death. PRE-084 administration promotes neuroprotection and neurite elongation through activation of protein kinase C (PKC) on motoneurons in an *in vitro* organotypic model of excitotoxic lesion [168]. Moreover, administration of PRE-084 significantly prevented the marked death of spinal motoneurons after spinal root avulsion in adult rats, an effect that was associated with attenuating ER stress within motoneurons and promoting the expression of GDNF by surrounding glial cells [93]. Remarkably, treatment of SOD1 mice with Sigma-1R agonists resulted in significantly improved motoneuron function and preservation, and increased animal survival [94, 152, 169, 170]. Several mechanisms have been hypothesized to underlie motoneuron protection in ALS models (Fig. 16.2). Sigma-1R agonists administration resulted in increased PKC-specific phosphorylation of NR1 subunits present in spinal motoneurons, likely reducing the calcium permeability of NMDA receptors and its influx into motoneurons, thereby attenuating excitotoxicity [94, 111]. Sigma-1R agonists, such as SKF10097 and PRE-084, have been reported to also suppress NMDA currents in rat retinal ganglion cells and cortical neurons through a PKC-dependent mechanism, leading to reduction of calcium influx into the cytoplasm [111, 166]. Sigma-1R agonists administration also reduced microglial and astroglial reactivity in the mutant SOD1 and in the wobbler ALS mouse models, and enhanced glial expression of neurotrophic factors, such as BDNF [94, 152]. In this sense, Sigma-1R activation has been linked to modulation of multiple aspects of microglial activation *in vitro* [171, 172], as well as to increase the glial expression of neurotrophic factors after spinal root avulsion [93].

Overall, two main interconnected mechanisms are likely to underlie the direct effect of Sigma-1R manipulation on motoneurons: the modulation of the neuronal excitability and the calcium homeostasis. The Sigma-1R is located in C-terminals in close proximity to Kv2.1 and SK channels, which appear as two suitable candidates for the Sigma-1R modulation of postsynaptic excitabil-

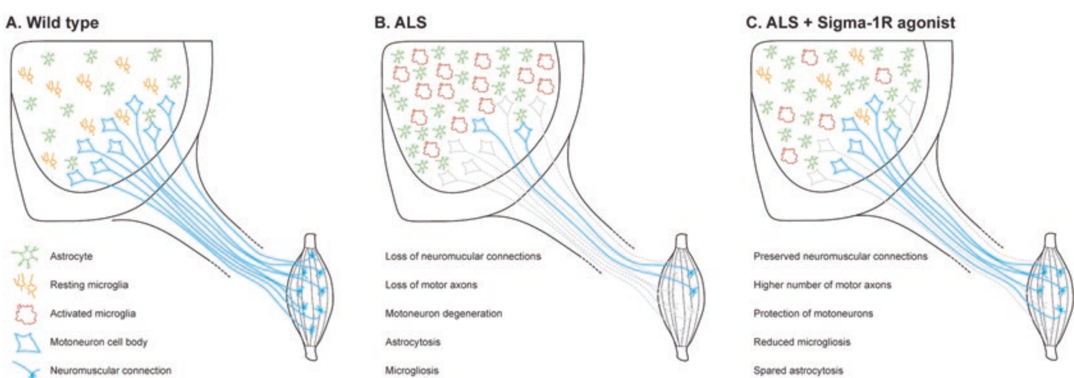

Fig. 16.2 Schematic representation of the effect of agonizing the Sigma-1 receptor in ALS mouse models. (**a**) Wild type spinal cord motoneurons project their axons from the anterior horn of the spinal cord through the anterior root to reach the skeletal muscles. (**b**) ALS spinal cord suffers a dramatic death of motoneurons, accompanied by loss of neuromuscular connections and ventral root motor axons. In addition, non-neuronal cells proliferate and become activated across the spinal cord, contributing to the disease progression. (**c**) Sigma-1 receptor agonists are able to prevent the loss of neuromuscular connections and motor axons, as well as the death of motoneuron cell bodies in the spinal cord. Furthermore, Sigma-1R agonists reduce microglial reactivity, despite no changes are observed in astrocytosis

ity of motoneurons. A body of evidence indicates that inhibition of m2AChR and/or activation of Kv2.1 and/or SK channels in C-terminals contribute toward reduction of motoneuron excitability [124, 133, 139]. Although the mechanisms by which Sigma-1R activates Kv2.1 and/or SK channels and thus decreases motoneuron excitability are still unclear, it has been shown within other systems that Sigma-1R can modulate activities of SK channels and a variety of Kv type channels [99, 142, 173]. Sigma-1R can form complexes with a variety of G-protein coupled receptors (GPCRs) that can subsequently alter ionotropic receptors including opioid and dopaminergic D1 receptors [174, 175] (Fig. 16.1).

As previously mentioned, Sigma-1R is located in the subsurface cisternae of C-terminals underlying the plasma membrane of motoneurons [89, 120]. Such physical proximity between the plasma membrane and the subsynaptic cisternae in C- terminals (less than 10 nM) makes direct molecular interaction possible for proteins located in adjacent membranes. Indeed, the Sigma-1R is characterized by a unique mode of action in regulating both the calcium entry at the plasma membrane level (e.g. via potassium channels, voltage-sensitive Ca^{2+} channels, etc.) and calcium mobilization from the endoplasmic stores (e.g. via IP_3 receptors). The ER supplies calcium directly to mitochondria via inositol 1,4,5-triphosphate receptors (IP_3 receptors) at close contacts between the two organelles referred to as mitochondrial-associated ER membranes (MAM). Sigma-1R is a calcium-sensitive and ligand operated chaperone at MAM, normally forming a complex with another chaperone, binding immunoglobulin protein (BiP), which normally prevents the Sigma-1R from translocation. Upon ER calcium depletion or via ligand stimulation, Sigma-1R dissociates from BiP, leading to prolonged calcium signaling into mitochondria via IP_3 receptors. Sigma-1R translocation has been shown to occur under chronic ER stress conditions. Indeed, increasing Sigma-1R in cells counteracts ER stress response, whereas decreasing its expression enhances apoptosis [90]. Subsequently, activity of both Kv2.1 and SK channels has been shown to be modulated by calcium, either directly or indirectly through Ca/calmodulin/calcineurin dependent mechanisms [176, 177] (Fig. 16.1).

In addition, Sigma-1R also contributes to maintenance of protein quality by regulating protein degradation and stability. Indeed, abnormal Sigma-1R accumulation is found in neuronal nuclear inclusions in neurodegenerative diseases [151, 178]. Sigma-1R participation in the degradation of misfolded protein via the ER machinery linked to the ubiquitin-mediated UPR suggests that Sigma-1R may function to counteract this pathological mechanism and promote survival in affected motoneurons. Ligand activation may promote and stabilize Sigma-1R oligomers, thus conferring improved chaperone functionality to the receptor [90].

Finally, modulation of Sigma-1R may also contribute to neuroprotection by reducing oxidative stress. It was shown that depletion of Sigma-1R leads to increased oxidative stress and abnormal mitochondrial membrane potential, thus triggering cytochrome C release and elevated caspase-3 cleavages [179].

16.7 Bases of Motoneuron Vulnerability

Understanding the bases of motoneuron vulnerability is crucial for developing novel strategies to cope with MND. In this section we focus on those aspects of motoneuron vulnerability that are related to mechanisms in which Sigma-1R plays a relevant role: the alteration of excitability properties of motoneurons and calcium homeostasis. As previously mentioned, ALS is a degenerative disease in which lower and upper motoneurons are selectively vulnerable, but interestingly some groups of motoneurons are relatively resistant to the disease process. It has been hypothesized that the differential susceptibility of motoneuron populations might be related to their excitability properties. Indeed, a consistent clinical feature of ALS is the preservation of eye movements and the external sphincters function. Pathological studies confirmed that there is relative sparing of the cranial motor nuclei of the

oculomotor, trochlear and abducens nerves, and of the Onuf's nucleus in the sacral spinal cord, which innervates the external sphincter of the pelvic floor [180]. Although neuronal numbers are relatively well-preserved in these resistant motor nuclei, some pathological changes resembling those observed in ventral spinal cord motoneurons are present, but to a lesser degree [181, 182]. Oculomotor nuclei are also relatively spared in mutant SOD1 mouse models [183]. The pattern of innervation of extraocular muscles is different from other skeletal muscles. Neuromuscular junctions are distributed throughout the fiber length at a high density [184], and show some structural peculiarities [185]. About 20 % of the extraocular muscles fibers are innervated by multiple neuromuscular junctions [186]. Oculomotor motor units are amongst the smallest seen in any skeletal muscle [187], with high firing discharge rates. Even in the primary position of gaze, 70 % oculomotor neurons are active, commonly discharging at 100 Hz [188]. In contrast, there is strong experimental evidence of a special susceptibility of large, phasic motoneurons in the degenerative process of ALS. Electromyographic analysis performed in ALS patients revealed that the larger and stronger motor units are clearly more affected by the disease [189], and histopathological studies have described a preferential degeneration of large motoneurons in ALS [190]. In mutant SOD1 models, selective vulnerability of large fast-fatigable hindlimb motor units before the onset of clinical symptoms was reported, followed by affectation of fast fatigue-resistant motor units at symptoms onset, but with sparing of slow motor units [191]. This is consistent with the rapid denervation of extensor digitorum longus muscle (rich in fast fatigable motor units) and the resistance of soleus muscle (with mainly slow motor units) described along disease progression in $SOD1^{G93A}$ mice [192, 193].

Understanding the differences in properties of vulnerable vs. resistant motoneurons may provide insights into the mechanisms of neuronal degeneration, and identify targets for therapeutic manipulation. In an interesting study Brockington et al. [194] performed a microarray analysis to compare the gene expression profile of isolated motoneurons from the ALS-resistant oculomotor nuclei and ALS-vulnerable spinal cord motoneurons from post-mortem ALS patients tissue. They found nearly 2000 genes differentially expressed by the two motoneurons subtypes, participating in synaptic transmission, ubiquitin-dependent proteolysis, mitochondrial function, transcriptional regulation, immune system functions and the extracellular matrix. They further focused on glutamate and GABA neurotransmission. The AMPA glutamate receptor consists of four subunits, GluR1–GluR4, and the presence of the GluR2 subunit determines the calcium permeability of the receptor. In the absence of GluR2, the AMPA receptor–ion channel complex becomes permeable to calcium. Gene array results showed up-regulation of the GluR2 subunit in resistant oculomotor motoneurons relative to the vulnerable lumbar motoneurons, thus reducing calcium influx into the cells. On the other hand, GABA is the most widely distributed inhibitory neurotransmitter in the CNS and acts through the interaction with GABA-A (ligand-gated chloride channel) and GABA-B (metabotropic) receptors. In oculomotor motoneurons, there is up-regulation of six GABA-A receptor subunits and of GABA-B receptor subunit 2 relative to spinal motoneurons, leading to an increased inhibition. Other studies performed in mSOD1 models confirmed these findings, revealing an excitatory/inhibitory imbalance affecting synaptic inputs into spinal motoneurons [23]. To test the hypothesis that inhibitory interneuron innervation of motoneurons was abnormal in ALS, Chang and Martin [195, 196] measured GABAergic, glycinergic and cholinergic immunoreactive terminals on spinal motoneurons of $SOD1^{G93A}$ mice. They found reduction of glycinergic innervation from pre-symptomatic age (8 weeks), before loss of choline acetyltransferase-positive boutons, whereas no significant differences in GABAergic boutons density were found along age.

Interestingly, the increased excitation and reduced inhibition onto motoneurons has been hypothesized as a protective compensatory reaction rather a detrimental phenomenon [197]. As

above mentioned, oculomotor nucleus motoneurons are strongly resistant to degeneration, but have particular physiological characteristics, including high discharge rates [188]. In turn, vulnerable fast-fatigable spinal motoneurons are those with larger cell bodies and more phasic activity pattern. Surprisingly, early administration of an AMPA receptor agonist protected spinal motoneurons whereas an AMPA receptor antagonist enhanced motoneurons pathology in SOD1^{G93A} mice [197]. Furthermore, the authors proposed that reduction of gephyrin (an inhibitory synapse marker), increase of serotonin labeled area in the ventral spinal cord and increased C-boutons size and number are protective compensatory reactions that promote motoneuron survival. In agreement with these findings an abnormal response of the potassium-chloride co-transporter 2 (KCC2) in mutant SOD1 motoneurons in response to axonal damage and deafferentation [198] was recently described. KCC2 is a transmembrane chloride extruder that maintains low intracellular chloride levels, thereby allowing GABA and glycine to exert inhibitory transmission during adulthood [199–201]. Under normal conditions, KCC2 is down-regulated after motoneuron insults thus promoting increased excitability needed for axonal regeneration [202, 203]. In contrast, mutant SOD1 motoneurons were unable to down-regulate their KCC2 and thus did not become hyperexcitable even when already disconnected from their muscles [198]. Further studies revealed that functional overload is able to rescue motor units in mutant SOD1^{G93A} mice [204], supporting the hypothesis of hypoexcitability as one potential factor underlying selective motoneuron damage.

In vitro studies of motoneuron excitability also show discrepancies regarding whether hypo- or hyperexcitability is a susceptibility factor for motoneurons in ALS. Changes in excitability have been reported to occur very early in mutant SOD1 mice [205]. Motoneurons from mutant SOD1 embryos recorded in culture show signs of hyperexcitability [206, 207], as well as motoneurons in *in vitro* preparation of mutant SOD1 embryonic spinal cords [208] or from the hypoglossal nucleus in the brainstem [209]. Contrarily, Pambo–Pambo et al. [210] did not observe any change in spinal motoneurons excitability properties, whereas Bories et al. [211] and Leroy et al. [212] reported spinal motoneurons to be hypoexcitable. A note of caution must be taken within this context since most of these studies were performed at developmental stages, when the maturation of the spinal circuitry is not yet completed.

Motoneurons express low levels of cytosol calcium-binding proteins compared to other neuronal populations, with motoneuron populations that are typically lost earlier during the disease course showing the lowest expression levels, suggesting that reduced cytosol calcium buffering contributes to the selective vulnerability of motoneurons [213, 214]. In fact, ALS-vulnerable spinal and brainstem motoneurons display low endogenous Ca^{2+} buffering capacity, 5–6 times lower than that of ALS-resistant motoneurons (i.e. oculomotor motoneurons), making them more susceptible to excitotoxic insults [215]. However, this view may not agree with the above mentioned oculomotor motor units properties since, although this motoneuron population is highly active, it is not vulnerable to ALS.

Interestingly, novel evidence has recently pointed out the potential contribution of C-boutons as participating in ALS pathophysiology [127, 140]. As described in Sect. 16.4, the postsynaptic membrane of C-boutons is rich in numerous proteins, including Sigma-1R [135], M2 muscarinic receptors [129–131], voltage-gated Kv2.1 [132] and Ca2+−activated K (SK) channels [133], connexin 32 [134], VAMP-2 [129], and neuregulin-1 [136]; whereas the presynaptic element contains, at least, neuregulin-1 receptors ErbB2 and ErbB4 [136]. Several alterations that may be related to C-bouton have been reported in ALS. It has been shown that mutations in Sigma-1R cause juvenile ALS [149, 150]. In agreement with this observation, knocking down Sigma-1R in mutant SOD1 mice leads to reduced lifespan [140], whereas treatment with a Sigma-1R agonist is neuroprotective [94]. Other morphological alterations appear to be present in ALS-linked mutations of VAMP-associated pro-

tein B, which is abnormally aggregated in C-boutons altering their function (VAPB, ALS8) [216]. The neuregulin1/ErbB system is also involved in ALS pathogenesis since ErbB4 mutations leading to a reduced autophosphorylation of ErbB4 receptors are associated with a hereditary late onset form of ALS [145], and neuregulin1/ErbB signaling alterations have been also observed in $SOD1^{G93A}$ mice [144].

16.8 Conclusions

Overall, mutations of Sigma1-R have been reported in ALS in human patients, and sigma-1R modulation has proven to protect motoneurons *in vitro* and in *in vivo* models of traumatic injury to motoneurons and neurodegeneration. Although the exact molecular mechanisms underlying such effect have not been elucidated yet, Sigma-1R is a pleiotropic target, involved in several functions, many of them related to the pathophysiology of MND, including modulation of neuronal excitability, calcium homeostasis, and ER and mitochondrial activity. Thus, the multi-functional nature of the Sigma-1R provides an attractive target for treating ALS. Further human trials will be needed to assess whether pharmacologically targeting Sigma-1R is a suitable tool to protect motoneurons in MND.

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The Sigma-1 Receptor–A Therapeutic Target for the Treatment of ALS?

17

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Abstract

The membrane bound 223 amino acid Sigma-1 Receptor (S1R) serves as a molecular chaperone and functional regulator of many signaling proteins. Spinal cord motor neuron activation occurs, in part, via large ventral horn cholinergic synapses called C-boutons/C-terminals. Chronic excitation of motor neurons and alterations in C-terminals has been associated with Amyotrophic Lateral Sclerosis (ALS). The S1R has an important role in regulating motor neuron function. High levels of the S1R are localized in postsynaptic endoplasmic reticulum (ER) subsurface cisternae within 10–20 nm of the plasma membrane that contain muscarinic type 2 acetylcholine receptors (M2AChR), calcium activated potassium channels (Kv2.1) and slow potassium (SK) channels. An increase in action potentials in the S1R KO mouse motor neurons indicates a critical role for the S1R as a "brake" on motor neuron function possibly via calcium dependent hyperpolarization mechanisms involving the aforementioned potassium channels. The longevity of SOD-1/S1R KO ALS mice is significantly reduced compared to SOD-1/WT ALS controls. The S1R colocalizes in C-terminals with Indole(ethyl)amine-N-methyl transferase (INMT), the enzyme that produces the S1R agonist, N,N′- dimethyltryptamine (DMT). INMT methylation can additionally neutralize endogenous toxic sulfur and selenium derivatives thus providing functional synergism with DMT to reduce oxidative stress in motor neurons. Small molecule activation of the S1R and INMT thus provides a possible therapeutic strategy to treat ALS.

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Keywords

Sigma-1 receptor • ALS • INMT • Motor neuron • Dorsal root ganglion • C-terminals

17.1 Summary of Biochemical and Pharmacological Properties of the Sigma-1 Receptor

The Sigma-1 receptor (S1R) was initially purified, cloned and reported by Hanner et al. (1) as a membrane protein of 223 amino acids. A crystal structure of the S1R derived in vitro from nanodiscs has revealed a homotrimer with a single transmembrane (TM) helix at the N Terminus of each monomer. The single TMs are directed into the ER lumen (and thus outside the cell at the plasma membrane) with the majority of the mass of each monomer located in the cytoplasm normal to the plane of the bilayer and "anchored" to the membrane via amphipathic sequences [1]. When expressed in vivo however on the surface of Xenopus Oocytes [2] or in the endoplasmic reticulum (ER) of CHO cells, the N and C termini have been demonstrated to be on the same side of the membrane [3, 4] consistent with two transmembrane sequences. The S1R in its purified form in vitro [5, 6] exists in monomeric, tetrameric, hexameric/octameric and higher oligomeric forms. Mutagenesis of the amino acids in a unique GGXXG (amino acids 87–91) sequence completely abrogated the formation of S1R oligomers when assessed in vitro. When expressed in COS cells in vivo [7] the S1R showed a preponderance of higher oligomeric forms when the cells were incubated with the antagonist, haloperidol and a preponderance of dimers/monomers when the cells were incubated with the agonist (+)- pentazocine. The functional activities of the S1R are likely to be linked to S1R agonist and antagonist alteration of the equilibria between these various forms [6, 8]. This ligand-regulated equilibrium between oligomeric and monomeric forms of the S1R is reminiscent of the general mechanism of action of a non liganded family of small heat shock (sHSPs) protein chaperones such as alpha/beta crystallins that are inactive in their oligomeric forms (25 mers and greater) and active in the monomer/dimer forms upon elevation of cellular temperature [8]. Since, in the case of the S1R, the oligomeric state of the receptor can be altered by the presence of "antagonists" such as haloperidol [7], inhibitors of the S1R could be more accurately designated as "inverse agonists" [8]. The C-terminal sequence (approximately 110 amino acids) in either the dimeric and/or monomeric form(s) may determine the manner by which the chaperone functions of the S1R occur with various "client" protein [4, 9–12].

An impressive number of synthetic compounds have been synthesized and characterized as selective agonists or antagonists for the S1R, [3, 13, 14]. Several synthetic long alkyl chain-N-phenylpropylamines have been identified as S1R ligands, including the endogenous compounds, sphingosine and N, N′- dimethylsphingosine, [8, 15, 16]. Additionally, steroids such as dehydroepiandosterone (DHEA), progesterone and pregnenolone sulfate [17], and the trace amine, N, N-dimethyltryptamine (DMT) [18] have been linked to S1R functions.

17.2 Biological Functions of the S1R

The S1R is a multi-tasking chaperone protein involved in a broad cell range of signaling pathways and functions [3, 8, 19, 29]. The various functional links of the S1R to neurodegenerative and other human syndromes (diseases) are also summarized in recent reviews [8, 20]. Sigma-1 receptor knockout mice are fertile [21], viable and show no obvious behavioral phenotypes except for a diminished response to pain [22]. The dorsal root ganglia (DRG) of the WT mouse is exceptionally rich in S1Rs [23] presaging an

important role for the S1R in regulating pain pathways in the spinal cord. The S1R regulates several types of voltage gated ion channels [11, 12, 24–26] and G-Protein coupled receptors [27, 28], suppresses ER stress [4, 29], and regulates autophagic responses in tumor cells that are associated with the unfolded protein (UPR) stress response [30]. The S1R has also been shown to interact with important ER stress related regulators such as BIP, PERK, and IRE1 and thus, in part, can alleviate ER stress [4, 31, 32]. The S1R interacts with the IP3 Receptor 3 in specialized mitochondrial associated membranes (MAMs) and modulates calcium flow from the ER into mitochondria [4].

17.3 The Main Biological Features of Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease associated with reduced function and loss of spinal cord motoneurons (MN) [33, 34]. Mutations of multiple genes result in the establishment of ALS [35] including expansion of GGGGCC hexanucleotide repeats and mutations in superoxide dismutase-1 protein (SOD1), the TAR-DNA binding protein, TDP43, and the RNA-binding protein, FUS. ALS can occur in adult humans and occasionally in juveniles [36, 37]. Synapses involving all known major neurotransmitters innervate motor neurons (MNs) including unusually large synapses referred to as C- terminals [38]. C-terminals are cholinergic postsynaptic sites with a unique ultrastructure seen at the EM level as subsurface cisternae of endoplasmic reticulum adjacent to the plasma membrane (PM). The C-terminal synapses are large (approximately 2–7 micrometers in diameter) and located only on soma and proximal dendrites of MNs. Changes in C-terminal morphology have been reported in animal models of ALS and spinal cord injury [39–42]. There is no cure for ALS. The drug, riluzole, provides partial respite by reducing levels of the excitatory neurotransmitter, glutamate, from neuronal synapses [43, 44].

17.4 The S1R and ALS

High levels of Sigma-1 receptors are found in motor neurons (MNs) of the spinal cord and brainstem [45, 46]. Metals that induce toxicity at high levels such as selenium [47] and/or genetic mutations can result in the loss of MNs with associations to ALS. An E102Q autosomal-

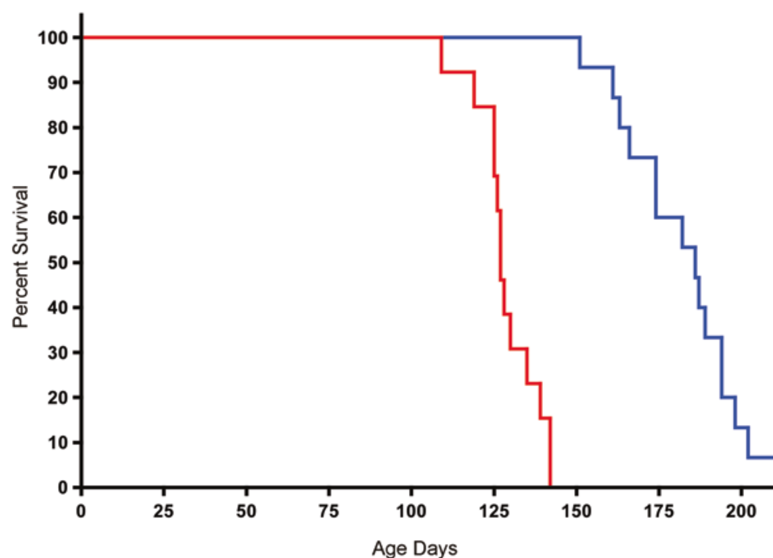

Fig. 17.1 Sigma-1 receptor slows ALS progression. Kaplan-Meyer end stage curves showing exacerbation of ALS in sigma-1 receptor deficient ALS SOD-1 mice (*red*/*left* curve) compared to ALS SOD-1 WT controls that contained the sigma-1 receptor (*blue*/*right* curve) . Median survival of mice was 186.0 days for ALS S1R WT mice and 127.0 days for ALS S1R KO mice. $p < 0.0001$; $\chi^2 = 32.29$ (Modified with copyright permission from: Mavlyutov et al. [5])

recessive mutation in the S1R results in juvenile ALS [36]. Overexpression of the E102Q S1R in Neuro2A cells leads to aggregation of the mutant S1R followed by reduction in mitochondrial ATP production and mislocalization of the TAR DNA binding protein, TDP43, from the nucleus to the cytoplasm. This effect of E102Q mutation could be rescued by addition of methyl pyruvate to maintain mitochondrial ATP production [48]. A monoallelic mutation in the 3′ untranslated region of the sigma-1 receptor gene in humans resulted in ALS and frontotemporal lobar degeneration (FTLD) [49]. In SOD1 ALS model mice MNs lose their shape, decrease in size, and show decreases in synaptic coverage [5]. Abundant S1R in the diseased MN that survive after fixa-

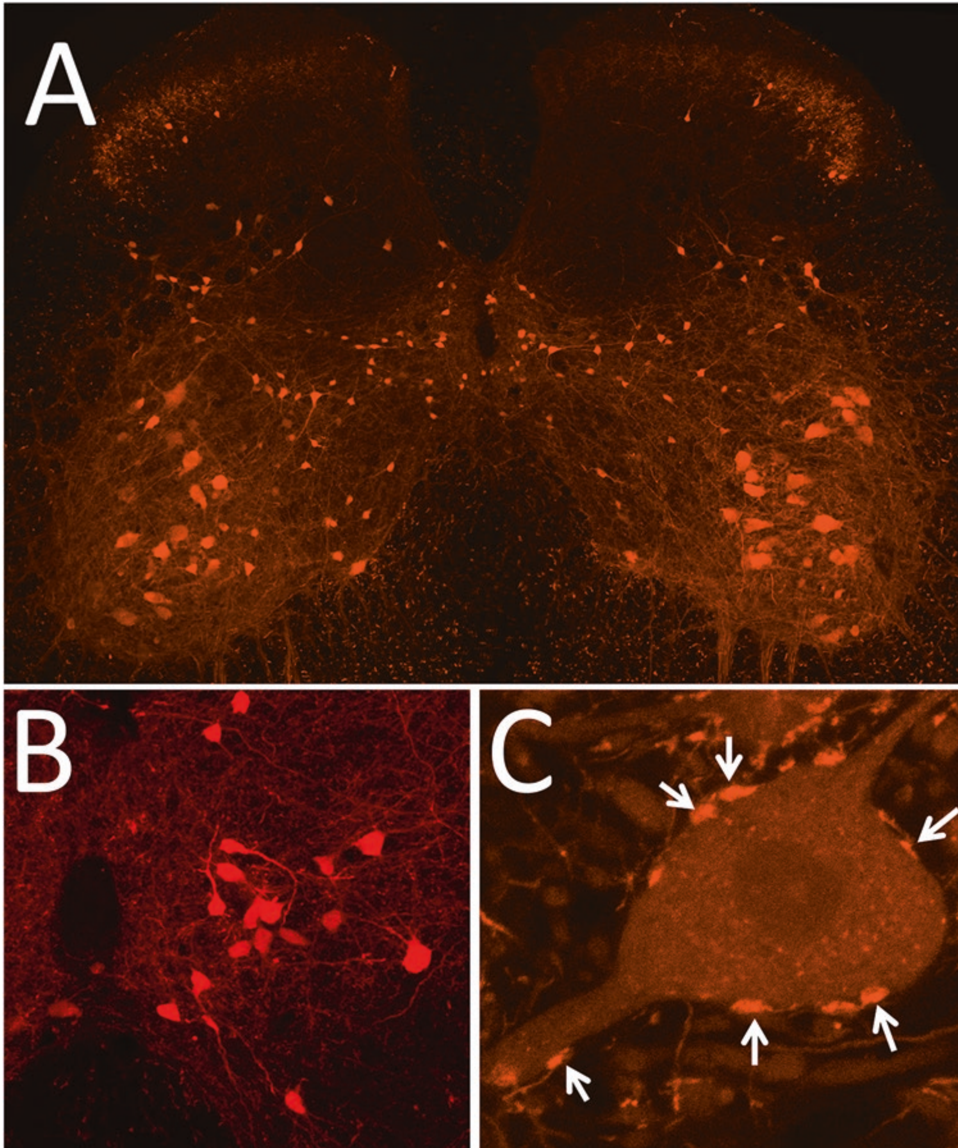

Fig. 17.2 Wild type (WT) mouse spinal cord identifying cholinergic neurons (fluorescent protein driven by the choline acetyl transferase (ChAT) promoter). (**a**) Cross section of spinal chord. (**b**) cholinergic neurons in laminae VII & X, close to the central canal give rise to C-boutons on MN. (**c**) MN. Covered by C-boutons (*arrows*)

tion have been observed but the total number of Sigma-1 receptors in the spinal cord are reduced because of MN death. S1R knockout mice on a Superoxide Dismutase-1 (SOD-1) ALS background demonstrated a faster onset of disease and decreased longevity [5] (Fig. 17.1). Application of S1R ligands significantly extended the lifespan of ALS model mice [50–52].

17.5 Subcellular Co localization of the S1R and INMT

A high density of cholinergic neurons occur in the mouse spinal cord (Fig. 17.2a) that arise from interneurons that are close to the central canal (Fig. 17.2b). These cholinergic interneurons synapse with the MNs to form the large C-boutons (Fig. 17.2c). A 3-dimensional reconstruction of the distribution of the S1R on the post synaptic side of the wild type mouse C- terminal (Fig. 17.3) shows precise juxtaposition of the S1R with the area of the presynaptically innervated bouton. The S1R is localized mainly to subsurface cisternae in C-terminals [53]. Significantly, the enzyme Indole(ethyl)amine-N-Methyl Transferase (INMT) that produces the S1R agonist N,N′ dimethyltryptamine (DMT) [18] and that can also detoxify endogenous alkyl sulfur and selenium derivatives [54, 55] co localizes with the S1R in post synaptic regions of primate C-terminals (Fig. 17.4). Toxic selenium has been noted to be causative for ALS symptoms, perhaps due to increased cellular oxidative stress [47]. As indicated previously, C-terminals also contain muscarinic type 2 acetylcholine receptors (M2AChR), voltage gated potassium channels (Kv2.1) and slow potassium (SK) channels located in the postsynaptic plasma membrane [53]. The presence of subsurface cisternae in postsynaptic densities correlates with postsynaptic hyperpolarization [56–58] that is generally mediated via Kv2.1 and SK channels in mammalian cells. Miles et al. [59] have shown in MNs that activation of M2AChR results in inhibition of SK channels, a result that reduces after hyperpolarization and thus increases neuronal excitability. We have shown that the excitability of MNs is higher in S1R knockout mice than in

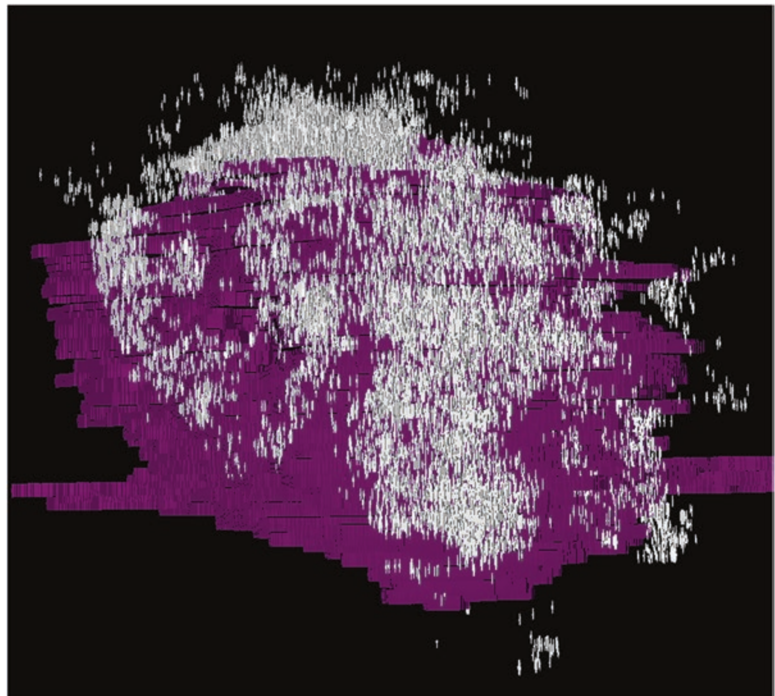

Fig. 17.3 A 3-D reconstruction of postsynaptic localization of sigma-1 receptor (*white/light*) juxtaposed to the presynaptic C- bouton (*purple/dark*). WT mouse spinal cord was fixed in 4 % paraformaldehyde, 0.1 % Glutaraldehyde. Sixty (60) micron thick sections were then sectioned on a vibrotome and processed for detection of sigma-1 receptor at EM level using a S1R specific antibody. Images of serial sections were generated and aligned together for 3D reconstruction in ImageJ soft- ware with TrakEM2 plugin [78]

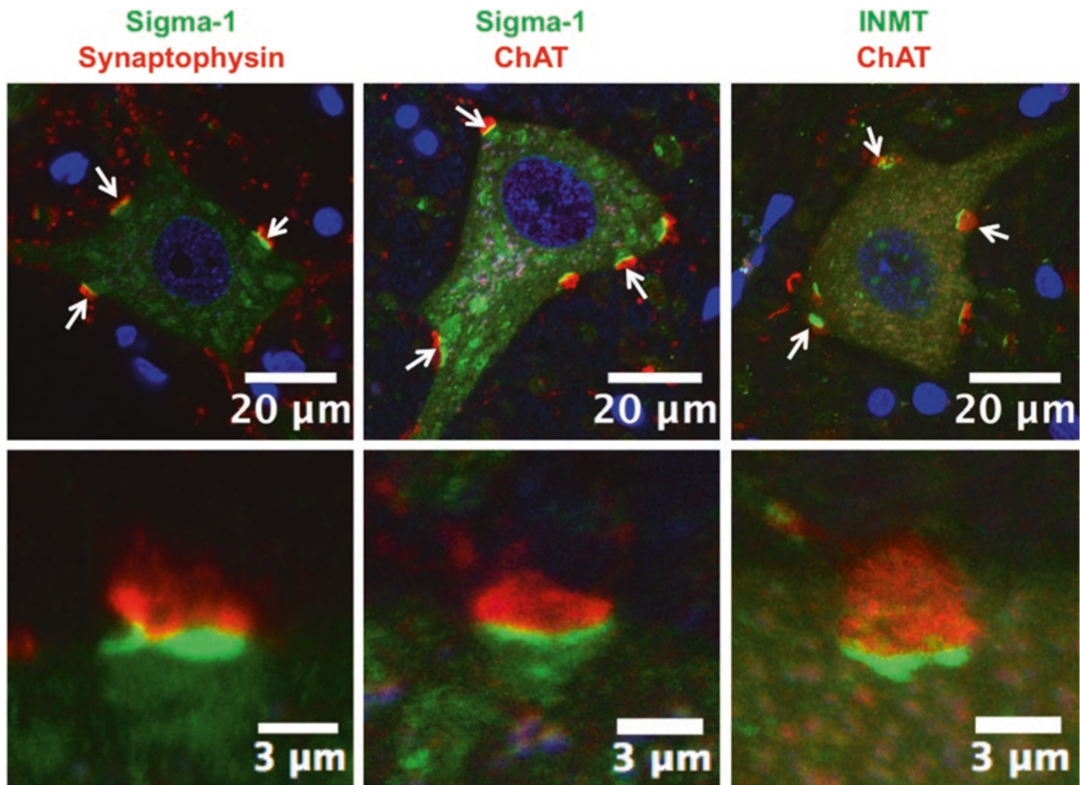

Fig. 17.4 Confocal images of immunolocalized sigma-1 receptor (*green* signal in the *left* and *middle* panels) and the DMT producing enzyme INMT (*green* in the *right* panel) to postsynaptic sites of C-terminals juxtaposed to presynaptic cholinergic (ChAT positive) boutons (*red*) in primate MNs . To demonstrate that the sigma-1 receptor is juxtaposed only to cholinergic postsynaptic densities of MNs, double labeling was performed with antibodies against synaptophysin (a universal marker for different types of chemical synapses). All synaptophysin-positive synapses are juxtaposed to the sigma-1 receptor. Blue (DAPI stain) indicates cell nuclei (Modified with copyright permission from Mavlyutov et al. [53])

their wild type counterparts that is consistent with the idea that a "brake" function normally occurs in the presence of the S1R probably through activation of Kv2.1 and/or SK channels [5] (Fig. 17.5). This idea is further supported by the fact that many potassium channels are modulated by the S1R [2, 11, 60, 61].

17.6 Possible S1R Mechanism(s) for Protecting Motor Neurons

Subsurface ER cisternae have been considered as calcium capacitors [62]. It is unclear by what molecular mechanisms the S1R could activate Kv2.1 and/or SK channels and thus decrease excitability. Physical proximity of the plasma membrane and the cisternae in C-terminals (distance of 10–20 nM) makes direct molecular interactions possible between the ion channels and with the S1R. This possibility is further supported by the fact that a majority of the mass of the S1R could reside on the cytoplasmic side of the ER cisternae membrane [1]. Activities of both Kv2.1 and SK channels are modulated by calcium, ether directly or indirectly through Ca/calmodulin/calcineurin mechanisms [63, 64]. In support of these general ideas, the S1R interacts directly with the inositol triphosphate type 3 receptor (IP3R3) to enhance calcium flow to mitochondria [4]. The dihydropyridine receptor

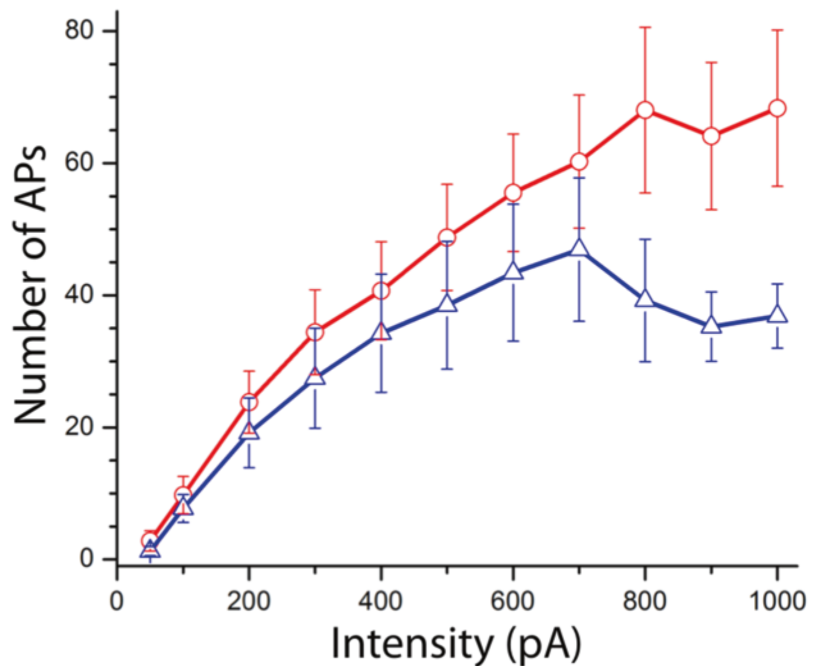

Fig. 17.5 Sigma-1 receptor slows ALS progression. Frequency-current relationships in motor neurons of SR1 KO mice (*open red circles*) and WT mice (*open blue triangles*). A significant increase in the slope of F-I relationship was apparent in SR1 KO mice at current intensities > 700 pA ($p < 0.05$). Bars are ± standard errors (Modified with copyright permission from Mavlyutov et al. [5])

in the plasma membrane of skeletal muscle directly interacts with the ryanodine receptor in ER cisternae. In cardiomyocytes the calcium channel, Orai, located in the plasma membrane and STIM-1 located in ER cisternae interact in a functionally relevant manner [65]. In these examples, such protein signaling interactions are important for enhanced calcium flow. It is thus reasonable to consider that modulation of potassium channels in C-terminals either directly or indirectly may underlie the mechanism(s) by which the S1R can reduce excitability of MNs.

Other possible mechanisms for S1R protection of MN certainly exist; for example, Since the S1R can also form complexes with a variety of G-protein coupled receptors (GPCRs) and can modulate their activities [27, 28], inhibition of M2AChR and consequent activation of Kv2.1 and/or SK channels in C-terminals should contribute toward reduction of MNs' excitability [59, 66, 67]. The growth factor Neuregulin-1 that protects MN loss in ALS is reduced in human ALS patients [68]. Neuregulin-1 co localizes with the S1R in C-terminal subsurface cisternae [69] expanding a possible role for S1R in MN as a Neuregulin-1 chaperone. The S1R has been also shown to regulate post-translational processing of growth factors [70].

In addition to S- adenosyl-L-methionine (SAM) dependent N-methylation of tryptamine, recombinant human INMT (hINMT) can also methylate endogenous thiol and selenium containing derivatives due to an alternate substrate thio ether methyl transferase (TEMT) enzyme activity [55, 71]. Methylation of alkyl sulfur, selenium and tellurium containing compounds via TEMT activity to produce trimethyl sulfonium (TMS), trimethyl selenonium (TMSe) and trimethyl telluronium (TMTe) is likely to occur for detoxification purposes [72] and for reduction of oxidative stress [73, 74]. These potential oxidative stress reducing properties of the TEMT activity of INMT is also in complete accord with previous demonstrations that the S1R reduces oxidative stress in cells [29, 75, 76]. Additionally Szabo et al. [77] have shown that DMT interacting with the S1R can robustly increase cellular survival by reducing hypoxic stress in cultured human cortical neurons (derived from induced pluripotent stem cells, iPSCs), monocyte-derived

macrophages (moMACs),and dendritic cells (moDCs) in a HIF-1 independent fashion. Taken together, the data support a mechanism whereby the co localization of S1R with INMT [53] in primate and human MNs is likely to provide protection in ALS through the S1R via both enzyme activities; that is, formation of DMT and reduction in oxidative stress via methylation of thiols and trace metals.

17.7 Conclusions

The S1R provides an attractive target for treating ALS. Therapeutic targeting of the S1R and INMT, using approaches specific to motor neurons, with selective S1R agonists and/or allosteric modifiers (in combination with other ALS therapies) is worthy of future endeavors.

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The Role of Sigma1R in Mammalian Retina

18

Jing Wang, Xuezhi Cui, Penny Roon, Alan Saul, and Sylvia B. Smith

Abstract

This review article focuses on studies of Sigma 1 Receptor (Sigma1R) and retina. It provides a brief overview of the earliest pharmacological studies performed in the late 1990s that provided evidence of the presence of Sigma1R in various ocular tissues. It then describes work from a number of labs concerning the location of Sigma1R in several retinal cell types including ganglion, Müller glia, and photoreceptors. The role of Sigma1R ligands in retinal neuroprotection is emphasized. Early studies performed *in vitro* clearly showed that targeting Sigma1R could attenuate stress-induced retinal cell loss. These studies were followed by *in vivo* experiments. Data about the usefulness of targeting Sigma1R to prevent ganglion cell loss associated with diabetic retinopathy are reviewed. Mechanisms of Sigma1R-mediated retinal neuroprotection involving Müller cells, especially in modulating oxidative stress are described along with information about the retinal phenotype of mice lacking Sigma1R (*Sigma1R*$^{-/-}$ mice). The retina develops normally in *Sigma1R*$^{-/-}$ mice, but after many months there is evidence of apoptosis in the optic nerve head, decreased ganglion cell function and eventual loss of these cells. Additional studies using the *Sigma1R*$^{-/-}$ mice provide strong evidence that in the retina, Sigma1R plays a key role in modulating cellular stress. Recent

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work has shown that targeting Sigma1R may extend beyond protection of ganglion cells to include photoreceptor cell degeneration as well.

Keywords
Retina • Sigma 1 receptor • Ganglion cell • (+)-pentazocine • ERG • Electroretinogram • nSTR • Negative scotopic threshold response • Mouse • Diabetic retinopathy • Photoreceptor cell • Müller cell • Ganglion cell death • Neuroprotection • rd10 mouse

18.1 Introduction

There has been substantial interest in the potential role of Sigma 1 Receptor (Sigma1R) as a target in neurodegenerative diseases. Numerous publications have explored this in brain and included in this compendium are chapters from several leaders in the field describing progress in the field. Neurodegenerative diseases affect not only the brain but also the retina, which some authorities consider an extension of the brain because the optic nerve is actually a tract of the brain. There have been a number of studies of this enigmatic protein in the retina and other components of the eye. This chapter will present an overview of some of investigations of Sigma1R in the eye with an emphasis on retina, the primary focus of work in our laboratory.

18.2 Structure and Organization of the Eye and Retina

The eye is situated within the bony orbit of the skull and functions to transmit visual information through the transparent cornea and lens to the retina. A diagram of the eye is shown in Fig. 18.1a. The cornea is the most anteriorly placed structure and is transparent to allow the passage of light. Light then travels through the aqueous humor and followed by the lens and ultimately strikes the photosensitive retina. Within the retina photic stimuli trigger a cis-to-trans isomerization of rhodopsin, the visual pigment, which in turn activates the neurochemical stimulation of retinal neurons for transmission via the optic nerve to the brain. Excessive exposure of the eye to light can damage various ocular structures including cornea, lens and retina. In addition to radiation, oxidative stress associated with ocular diseases such as diabetic retinopathy, glaucoma, macular degeneration, and cataract can trigger debilitating visual loss. The retina comprises the innermost tunic of the eyeball. Microscopically, the mammalian retina is composed of an outer pigmented layer, the retinal pigment epithelium (RPE), and an inner neurosensory layer, the neural retina. The retina is a stratified tissue characterized by cellular layers separated by synaptic layers. As shown in Fig. 18.1b, the outermost layer is the RPE. On its basal surface, which faces the choroid a major blood supply to outer retina, there are basal infoldings; whereas on its apical side, the RPE features microvillous processes, which interdigitate with the outer segments (OS) of photoreceptor cells. The OS are connected by a cilium to the inner segments (IS) of the photoreceptor cells, the first order neuron of the visual system. The photoreceptor nuclei form the outer nuclear layer (ONL). There are two general types of photoreceptor cells, rods and cones. Rods mediate scotopic (dark-adapted) vision, while cones mediate photopic (light-adapted vision). In the outer plexiform layer (OPL), the axons of rods and cones synapse with bipolar cells, the second order neurons of the visual pathway. The bipolar cells occupy the inner nuclear layer (INL). Within the INL are amacrine and horizontal cells, modulatory neurons of the retina. The major glial cell of the retina known as the Müller cell has its cell bodies within the INL, radially oriented processes emanate from these cells towards the inner and outer retina. Other glial cells of the retina include astrocytes and microglia. The inner plexiform layer (IPL) of retina is the synaptic layer in which bipolar cells communicate with ganglion cells (and some dis-

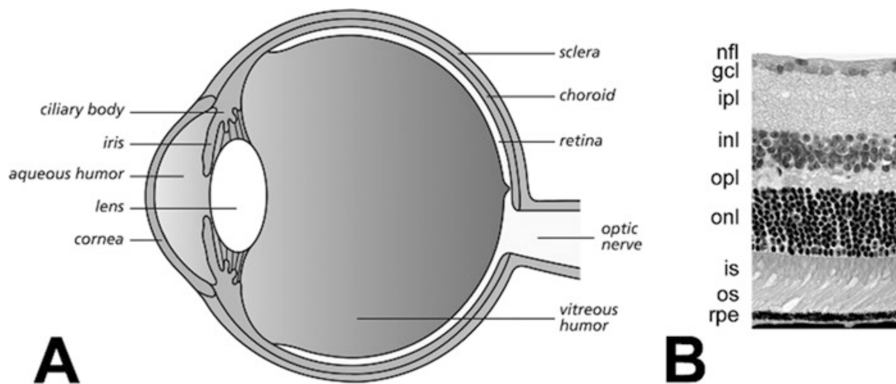

Fig. 18.1 *Anatomy of the eye and microstructure of the retina.* (**a**) Diagram of the mammalian eye showing the anteriorly placed cornea, behind which is the aqueous humor and the centrally placed lens. The ciliary body projects into the eyeball. It synthesizes aqueous humor and also has suspensory ligaments that hold the lens in place behind the iris. The retina is the innermost tunic of the eye and comprises the posterior five-sixths of this inner tunic. (**b**) Hematoxylin-eosin stained section of mammalian retina (mouse). The outermost layer is the retinal pigment epithelium (*RPE*). The microvillous processes of RPE cells interdigitate with the outer segments (*OS*) of adjacent photoreceptor cells. The cell bodies of the photoreceptor cells, known as rods and cones, constitute the outer nuclear layer (*ONL*). Photoreceptor cells synapse in the outer plexiform layer (*OPL*) with bipolar cells. Bipolar cells, horizontal cells and amacrine cells have their cell bodies in the inner nuclear layer (*INL*). Axons of the bipolar cells synapse in the inner plexiform layer (*IPL*) with dendrites of the ganglion cells (*gcl*). The axons of the ganglion cells form the nerve fiber layer (*nfl*); then continue on as the optic nerve. (*Antioxidants and Redox Signaling,* with permission, fig. 12, Ref. [65])

placed amacrine cells). The ganglion cells reside in the ganglion cell layer (GCL) (along with some amacrine cells). The axons of the ganglion cells form the nerve fiber layer (NFL), the fibers of which join together to form cranial nerve II, the optic nerve. The inner most layer of the retina, the inner limiting membrane is actually formed by the inner processes (footplates) of the radially oriented Müller cells and the outer limiting membrane is also formed by footplates of the Müller cells. Comprehensive information about the eye can be found in *Adler's Physiology of the Eye* [1] and details about retinal structure in health and disease are provided in an excellent three volume series *Retina* [2].

18.3 Establishing the Presence of Sigma1R in Retina

The existence of Sigma1R was proposed in the mid-1970s [3], however it would not be until the mid-1990s that Sigma1R would be investigated in eye. The first studies examined Sigma1R in cells of the lacrimal gland, which produces tears to keep the cornea moist. Schoenwald performed binding studies and demonstrated the presence of Sigma1R in rabbit lacrimocytes [4]. Bucolo and colleagues demonstrated the presence of Sigma1R binding in rabbit iris-ciliary body [5]. Within a few years, Sigma1R binding studies were performed in retina. Senda and colleagues showed that (+)-pentazocine ((+)-PTZ) and (+)-DTG bound bovine retinal membranes with high affinity and established that the densities of Sigma1R were higher in retina than in other tissues, including brain, adrenal medulla and lacrimocytes [6]. While the studies provided evidence that Sigma1R were present in retina, they did not demonstrate in which cell type(s) Sigma1R was present nor did they establish the molecular identify of the receptor. Working with our colleague, Dr. Vadivel Ganapathy, whose laboratory cloned Sig1R in human [7], rat [8] and mouse [9] at the same time as Hanner's lab did so in guinea pig [10], we addressed these issues in mouse retina. Using reverse transcription–polymerase chain reaction (RT–PCR) analysis we amplified Sigma1R in neural retina, RPE–choroid complex, and lens isolated from mice [11]. We then

determined in which retinal layers *Sigma1R* gene was expressed using *in situ* hybridization analysis and found abundant expression in the ganglion cell layer, cells of the inner nuclear layer, inner segments of photoreceptor cells and the RPE. We used an antibody developed in the lab of P. Casellas [12] and detected the Sigma1R protein in retinal ganglion, photoreceptor, RPE cells and surrounding the soma of cells in the inner nuclear layer. This study provided information on the cellular location of Sigma1R in retina and established the molecular identity of Sigma1R in retinal cell lines (rMC1, ARPE19 and RGC-5). In this same study, we demonstrated the presence of Sigma1R in lens and corneal epithelial cells and confirmed the presence of Sigma1R in the iris-ciliary body. Other laboratories have investigated the location of Sigma1R in retina. Liu and co-workers used RT-PCR and immunofluorescence to localize Sigma1R in the inner nuclear and ganglion cell layer of the rat retina [13]. They determined that Sigma1R was present in horizontal cells and several types of amacrine cells. Interestingly, they did not observe Sigma1R labeling in rat bipolar cells. Elegant ultrastructural studies from the Guo lab have demonstrated Sigma1R in mouse bipolar, photoreceptor and ganglion cells [14] as described in detailed in this book.

18.4 Studies of Sigma1R Neuroprotective Functions *In Vitro*

The earliest examination of the neuroprotective role of Sigma1R in retina used minced tissues isolated from embryonic rats [15]. The mixed neuronal-glial culture was exposed to L-glutamate, a known neurotoxin, at a high concentration [500 μM] for 10 min and cell death was determined using the trypan blue exclusion viability assay. The cells were pre-treated 10 min prior to insult with Sigma1R agonists SA4503 or (+)-PTZ and both afforded neuroprotection in a dose-dependent manner. These studies laid the foundation of future work examining the role of various Sigma1R ligands in a variety of retinal *in vitro* systems.

Some of the earliest *in vitro* studies of Sigma1R in retina were conducted in a cell line (RGC-5) that was originally described as a mouse retinal ganglion cell line [16], but was later determined to be the 661 W photoreceptor cell line derived from mouse [17]. Nonetheless, the early studies using these cells showed that targeting Sigma1R could prevent death of this transformed neuronal cell line and likely involved regulation of calcium channels [18, 19] and ER stress [20]. Bucolo demonstrated that increased intraocular pressure, which occurs in some forms of glaucoma, could be attenuated when Sigma1R ligands (+)-PTZ and (+)NANM (N-allylnormetazocine) were applied topically [5]. This group continued to study the beneficial effects of targeting Sigma1R in ischemia-reperfusion damage [21, 22], ocular hypotension models [23], and oxidative stress models [24]. In all cases targeting Sigma1R proved useful in attenuating stress-induced retinal cell loss.

18.5 Sigma1R in Retinal Disease: Expression Analysis and *In Vitro* Studies

The aforementioned studies set the stage to examine whether Sigma1R would continue to be expressed during retinal disease. We were particularly interested in its expression during diabetic retinopathy (DR) because ganglion cells, which our studies showed express Sigma1R at high levels [11], are lost in DR in human patients [25], rats [25] and mice [26]. DR is a major sight-threatening disease and a leading cause of blindness globally [27]. It is characterized by loss of retinal neurons and disruption of vasculature [28]. Patients with diabetes lose color and contrast sensitivity within 2 years of onset. Focal ERG (electroretinogram) analysis, which detects electrical responses of ganglion cells, reveals dysfunction of these cells early in diabetes [reviewed in 29]. We carried out studies in retinas of diabetic and age-matched control mice [30]. Mice that had been made diabetic using strepto-

zotocin, a compound that kills pancreatic beta cells, had blood glucose levels that were consistently greater than 300 mg/dl. We did not administer insulin in the study to avoid confounding interpretation of the data. The neural retinas expressed Sigma1R mRNA at levels comparable to age-matched controls as assessed by semi-quantitative RT-PCR. *In situ* hybridization studies showed that *Sigma1R* continued to be expressed in ganglion cells of diabetic mice; western blot analysis and immunohistochemistry showed that Sigma1R protein was present in ganglion cells of diabetic mice. These were very encouraging findings because they provided a rationale for using Sigma1R agonists to block ganglion cell death characteristic of diabetic retinopathy.

To begin exploring the neuroprotective effects of Sigma1R ligands in retinal disease we first performed *in vitro* studies. Using the retinal neuronal cell line (RGC5), we demonstrated that exposure of these cells to high dosages of the excitotoxic amino acid homocysteine, which has been implicated in some forms of glaucoma, induced cell death, could be attenuated by pre- and co-treatment with (+)-PTZ ([3 or 10 μM]) [31]. There were limitations associated with using the RGC-5 cell line. First, millimolar concentrations of excitotoxins (e.g. glutamate, homocysteine) were required to induce cell death in these cells, whereas ganglion cells are sensitive to micromolar concentrations of these *in vivo* (e.g. [15 μM] glutamate). Second, cell lines replicate in culture, which is not characteristic of neurons *in vivo*. Third, neuronal cell lines do not form neurite processes characteristic of neurons. For these reasons we optimized culture of primary ganglion cells using the two-step immunopanning procedure developed by Barres [32, 33]. We used micromolar concentrations of glutamate and homocysteine as neurotoxic agents to study effects of (+)-PTZ as a neuroprotectant [34]. Primary ganglion cells pre-treated 1 h with (+)-PTZ followed by 18 h co-treatment with 25 μM Glu and (+)-PTZ showed a marked decrease in cell death: (25 μM Glu alone: 50 %; 25 μM Glu/0.5 μM (+)-PTZ: 38 %; 25 μM Glu/1 μM (+)-PTZ: 20 %; 25 μM Glu/3 μM (+)-PTZ: 18 %). Similar results were obtained when cells were exposed to 50 μM D,L-homocysteine and were pre-/co-treated with 3 μM (+)-PTZ. As with the studies using glutamate as a neurotoxin, the expression of Sigma1R at the gene and protein level was not altered by (+)-PTZ, suggesting that its neuroprotective effects involve activation of Sigma1R rather than altered expression of the receptor [34].

In addition to affording protection to ganglion cells, (+)-PTZ treatment preserved the processes of these neurons. We used differential interference contrast (DIC) microscopy in the cells exposed to homocysteine or glutamate and observed shrunken cell bodies and contracted and disrupted, stubby neuronal processes (Fig. 18.2.). Cells pre-/co-treated with (+)-PTZ showed marked preservation of the neuronal processes. The cell bodies were similar to the control cells and the fibers emanated in complex arrays. Clearly, (+)-PTZ protected the cells against death and preserved their fibers. The stereoselective effect of (+)-PTZ for sigmaR1 was established in experiments in which (−)-PTZ, the levo-isomer form of pentazocine, had no neuroprotective effect on excitotoxin-induced ganglion cell death.

Our *in vitro* studies using primary retinal ganglion cells were complemented by studies in primary retinal Müller cells isolated from mouse retina. Müller cells, the key retinal glial cell, span the retinal thickness, contacting and ensheathing neuronal cell bodies and processes. They are crucial role for neuronal survival providing trophic substances and precursors of neurotransmitters to neurons [35]. Evaluating the role of Sigma1R in glial cells has great relevance to neurodegenerative diseases. Most retinal diseases are associated with reactive Müller cell gliosis, which may contribute to neuronal cell death. We sought to characterize Sigma1R in these cells. We used the rat Müller cell line, rMC-1 and showed that *Sigma1R* mRNA was expressed in these cells [36]. We then optimized primary mouse Müller cell isolation and culture in our lab, verifying that the cells were not contaminated by neurons or RPE cells [37]. In studies using primary Müller cells we showed that *Sigma1R* mRNA was expressed in these cells and determined using laser scanning confocal micros-

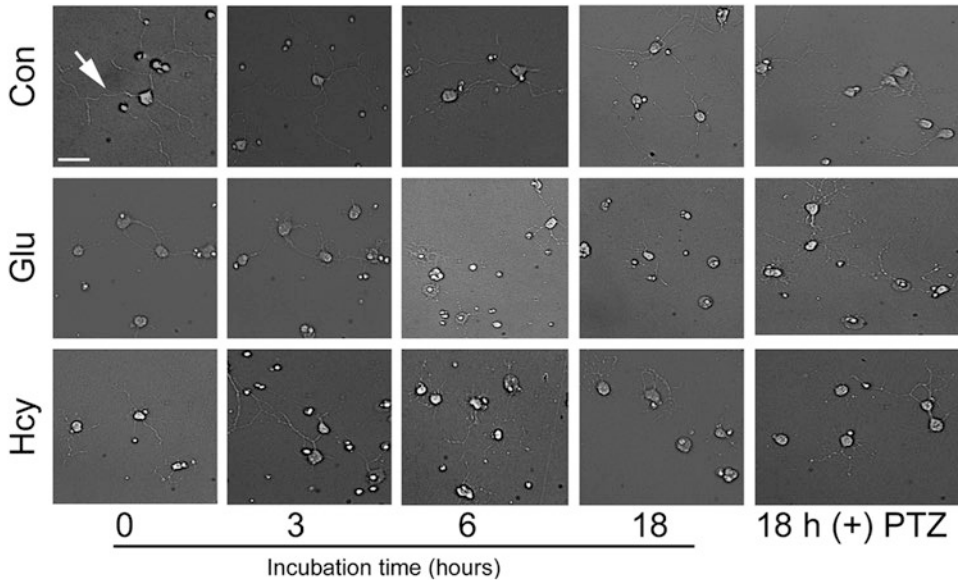

Fig. 18.2. *Differential interference contrast microscopic analysis of cells exposed to Glu or homocysteine (Hcy) and (+)-PTZ.* 1°GCs were isolated and cultured as described. Control cells (Con) were not exposed to excitotoxins; the row of photographs labeled "Glu" shows cells that were incubated with 25 μM Glu over a period of 18 h; photomicrographs were acquired at 0, 3, 6, 18 h post-incubation. The row of photographs labeled "Hcy" shows cells that were incubated with 50 μM Hcy over an 18 h period and photographed at 0, 3, 6 and 18 h post-incubation. In additional experiments, cells were pre-treated with (+)-PTZ for 1 h and then co-incubated with (+)-PTZ and the excitotoxin for 18 h. Cell bodies and processes of cells co-treated with either Glu or Hcy and (+)-PTZ were similar in appearance to control cells. In the *top left panel* (control, 0 time) the *arrow* points to a process extending from the cell body. (Magnification bar = 15 μm). All photomicrographs are the same magnification (*IOVS*, with permission fig. 8, Ref. [34])

copy that Sigma1R was present on the nuclear and endoplasmic reticular membranes of these primary glial cells [36] (Fig. 18.3). It is noteworthy that other investigators interested in determining the subcellular location of Sigma1R in retina have reported its location on the nuclear membrane of photoreceptor cells [14]. This work is described in more detail elsewhere in this book.

Unlike primary neurons, primary Müller cells can proliferate in culture (just as they can proliferate *in vivo*), which allowed us to analyze Sigma1R binding activity in these cells. Previous studies of Sigma1R in retina demonstrated binding activity; however these studies used whole retina from large models (bovine) [6] and did not attempt to study the binding activities of individual retinal cell types. Sigma1R binding in primary Müller cells was characterized using the high-affinity Sigma1R ligand (+)-PTZ [38]. The binding was saturable over a (+)-PTZ concentration range of 1.25–75 nM. The apparent dissociation constant (K_d) for primary Müller cells was 18.9 ± 5.6 nM. Scatchard analysis of the binding data revealed the presence of a single binding site in these cells. The binding constant (B_{max}) calculated for primary Müller cells was 1.32 ± 0.13 pmol/mg protein.

We also analyzed Sigma1R binding activity when Müller cells were exposed to either nitric oxide (NO) donors (SNAP, SNOG, SIN-1) or reactive oxygen species (ROS) donors (hydrogen peroxide and xanthine;xanthine oxidase), because NO and oxidative stress are implicated in retinal disease [39, 40]. Treatment of cells with NO and ROS donors resulted in marked increase in [^{3}H]-(+)-PTZ binding activity. Taken collectively, the data show that oxidative stress increases Sigma1R binding activity [38].

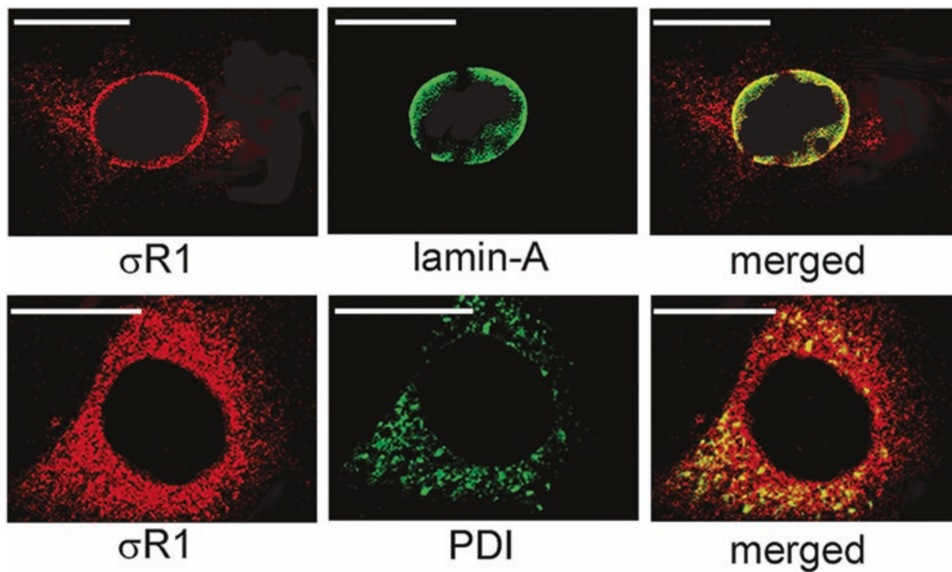

Fig. 18.3 *Subcellular localization of σR1 in1°MCs.* Müller cells were isolated from mouse retina and cultured. They were subjected to double-labeling immunocytochemical analysis using a polyclonal antibody specific for σR1 (which *fluoresced red*) and monoclonal antibodies (which *fluoresced green*) that label the nuclear membrane (lamin-A) or the endoplasmic reticulum (PDI), respectively. Optical sectioning by confocal microscopy detected co-localization of σR1 with lamin-A (merged image) and with PDI (merged image). In the merged images, the signal was *orange* when the *red* and *green fluorescence* overlap indicative of co-localization. (*IOVS*, with permission, fig. 2, Ref. [36])

18.6 Sigma1R in Retinal Disease: Expression Analysis and *In Vivo* Studies

Given the promising effects of (+)-PTZ in attenuating death of ganglion cells *in vitro* and the evidence that oxidative stress increases Sigma1R binding activity, we sought to investigate the effects of Sigma1R activation in murine models of diabetic retinopathy [41]. We used an induced diabetes model initially (streptozotocin injections in WT mice) to establish dosages and then performed a comprehensive analysis using the *Ins2*$^{Akita/+}$ mouse, which has a point mutation of the Insulin2 gene leading to hyperglycemia and hypoinsulinemia in heterozygous mice by ~4 weeks [26]. In addition to increased retinal vascular permeability and an increase in acellular capillaries, *Ins2*$^{Akita/+}$ mice demonstrate ~20–25 % reduction in the thickness of the inner plexiform layer (IPL), ~16 % reduction in the thickness of the INL, and ~25 % reduction in the number of cell bodies in the retinal GCL. Cells in the GCL are immunoreactive for active caspase-3 after 4 weeks of hyperglycemia, consistent with cell death by apoptosis. In our study, *Ins2*$^{Akita/+}$ mice were injected intraperitoneally beginning at diabetes onset with 0.5 mg kg^{-1} (+)-PTZ twice weekly for 22 weeks. The progression of changes in *Ins2*$^{Akita/+}$ retinas compared with wild-type over this time period is shown in Fig. 18.4. Wild-type mice had uniform thickness of layers throughout the central and midperipheral retina (Fig. 18.4a). *Ins2*$^{Akita/+}$ mice had modest INL thinning at 7 weeks (Fig. 18.4b) and more dramatic INL cell dropout at 10 weeks (Fig. 18.4c). By 17–25 weeks, there was marked INL and GCL cell loss in *Ins2*$^{Akita/+}$ mice (Figs. 18.4d–f). The IPL, which is composed of synaptic processes of cells in the INL and GCL, was also thinner. The cell loss and misalignment of inner retinal layers resulted in a somewhat wavy appearance in some of the retinas of 17- to 25-week-old *Ins2*$^{Akita/+}$mice. We found that (+)-PTZ treatment of *Ins2*$^{Akita/+}$ mice led to marked preservation of retinal architecture. The data shown (Figs. 18.4g–i) are from retinas

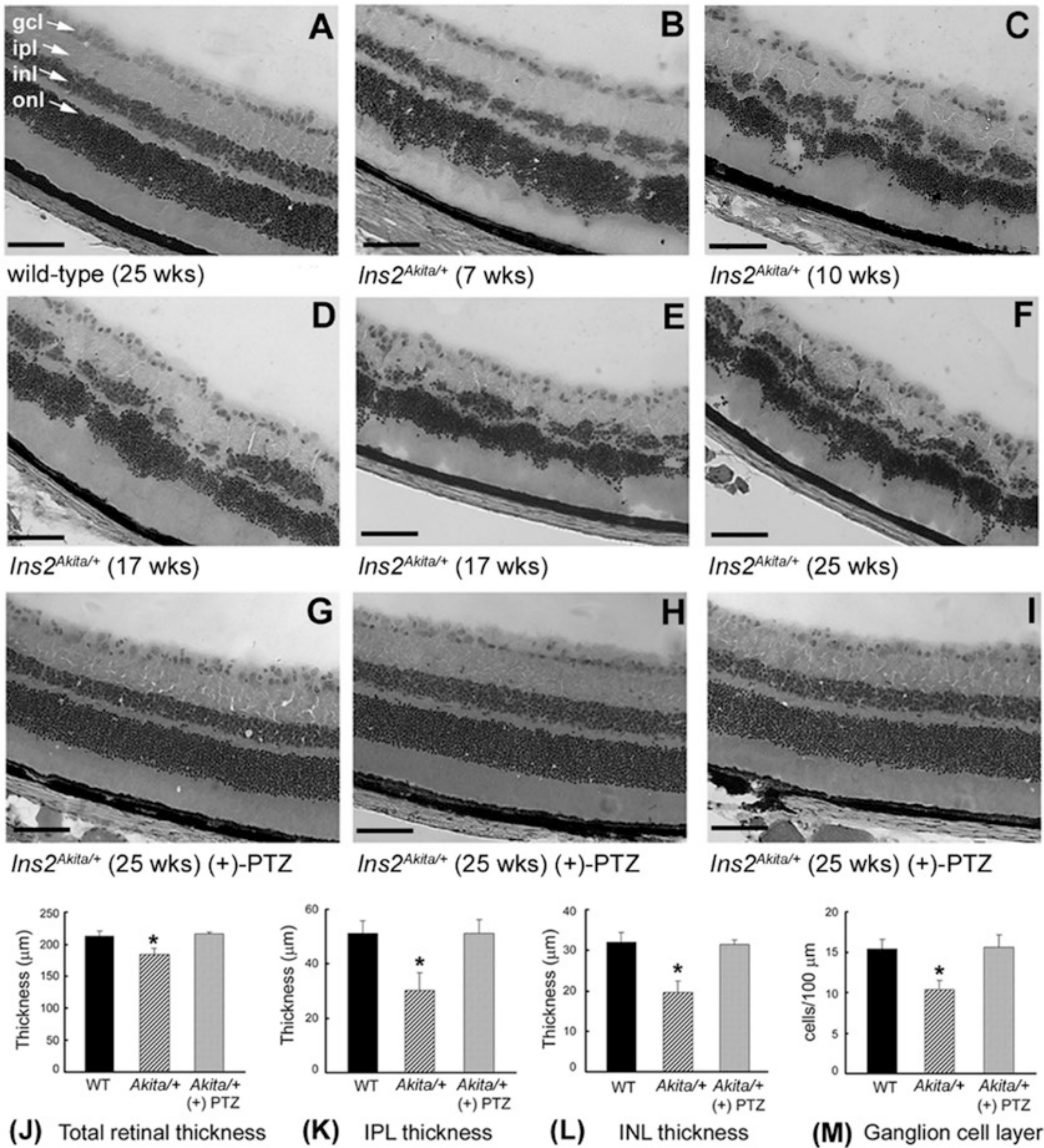

Fig. 18.4 *Preservation of retinal structure in Ins2$^{Akita/+}$ mice administered (+)-pentazocine.* Representative H&E-stained retinal cryosections of (**a**) wild-type mice: GCL cells are distributed evenly, nuclear layers are uniformly thick; (**b**–**f**) *Ins2$^{Akita/+}$* mice: INL becomes disrupted with age, GCL density is decreased; (**g**–**i**) *Ins2$^{Akita/+}$*mice treated with (+)-pentazocine (0.5 mg/kg, 2X/wk. i.p./22 weeks): marked preservation of retinal layers. *PTZ* pentazocine, *gcl* ganglion cell layer, *ipl* inner plexiform layer, *inl* inner nuclear layer, *onl* outer nuclear layer, magnification bar = 50 µm). Retinal sections were subjected to morphometric analysis: (**j**) total retinal thickness (**k**) IPL thickness (**l**) INL thickness (**m**) number of cell bodies in GCL per 100 µm length of retina. Data are means ± S.E. of measurements from retinas of 6 wildtype (12 eyes), 9 *Ins2$^{Akita/+}$* (18 eyes) and 8 *Ins2$^{Akita/+}$* treated with (+)-pentazocine (16 eyes). * Significantly different from wildtype and (+)-pentazocine-treated mice (p < 0.001). (*IOVS*, with permission, fig. 3, Ref. [41])

of three different (+)-PTZ treated *Ins2*$^{Akita/+}$ mice, representative of the excellent retinal structure observed in the eyes of all diabetic mice treated with (+)-pentazocine (n = 8 mice, 16 eyes). Morphometric analysis indicated a significant decrease in the thickness of *Ins2*$^{Akita/+}$ mouse retinas, whereas (+)-PTZ-treated *Ins2*$^{Akita/+}$ mice were comparable to wild-type mice (Fig. 18.4j). The IPL and INL in *Ins2*$^{Akita/+}$ mice measured 30.3 ± 6.4 and 19.68 ± 2.72 μm, respectively. In (+)-PTZ-treated *Ins2*$^{Akita/+}$ mice, the values for the thicknesses of the IPL and INL (51.2 ± 4.9 and 31.3 ± 1.3 μm, respectively) were comparable to those in wild-type mice (51.1 ± 4.6 and 31.9 ± 2.4 μm, respectively; (Fig. 18.4k, l). There were 30 % fewer cell bodies in the GCL of *Ins2*$^{Akita/+}$ mice compared with wild-type mice (10.4 ± 1.2 vs. 15.4 ± 1.2 cells/100 μm retinal length, respectively) whereas the values for (+)-PTZ-treated *Ins2*$^{Akita/+}$ mice (15.6 ± 1.5 cells/100 μm) were similar to those in wild-type (Fig. 18.4m). The (+)-PTZ-treated *Ins2*$^{Akita/+}$ mice remained hyperglycemic throughout treatment. Blood glucose levels were ~500 mg/dL (similar to untreated *Ins2*$^{Akita/+}$ mice) and were significantly higher than those in wild-type mice (104–160 mg/dL), suggesting that hyperglycemia *per se* may not be sufficient to trigger retinal neuronal loss in diabetes.

Recently, other studies have been conducted to evaluate whether targeting Sigma1R can afford retinal neuroprotection *in vivo*. Hara's group used cutamesine dihydrochloride, an agonist of Sigma1R and evaluated the effects of intravitreal administration on light irradiation-induced photoreceptor cell death. Cutamesine suppressed light-induced retinal dysfunction and thinning of the outer nuclear layer in the mouse retina [42]. These were important findings because they suggested that targeting Sigma1R may have potential in neuroprotection of non-ganglion neurons within retina. Very recently, we explored activation of Sigma1R in treatment of a genetic model of photoreceptor cell loss. We utilized *Pde6β*^{rd10} (*rd10*) mice, which harbor a mutation in the rod-specific phosphodiesterase gene *Pde6β* and lose rod and cone photoreceptors within the first six weeks of life, as a model for severe retinal degeneration. Systemic administration of (+)-PTZ beginning at post-natal day 14 and continuing every other day for several weeks led to significant rescue of cone function in in treated *rd10* mice as indicated by photopic electroretinographic recordings using natural noise stimuli and preservation cone cells upon retinal histological examination [43, 44]. The dramatic protective effect appears to be due to activation of Sigma1R because when *rd10/Sigma1R*$^{-/-}$ mice were administered (+)-PTZ, there was no preservation of cones [44]. (+)-PTZ treatment attenuated reactive gliosis and decreased lipid and protein oxidative stress in the mutant retinas. Additionally, activation of Sigma1R initially increased expression of the key antioxidant transcription factor NRF2 and downstream antioxidant genes, which then returned to WT levels over the course of the disease. The finding that activation of Sigma1R attenuates inherited photoreceptor cell loss may have far reaching therapeutic implications for retinal neurodegenerative diseases.

18.7 Mechanisms of Sigma1R Retinal Neuroprotection

Our *in vivo* data showing activation of Sigma1R might afford retinal neuroprotection prompted studies to understand the mechanism(s) of this neuroprotection. Pioneering work from Su's laboratory showed that Sigma1R acts as a ligand-operated molecular chaperone at the mitochondria-associated endoplasmic reticulum (ER) membrane [45–47]. The ER is the entry site for proteins into the secretory pathway. Proteins are translocated into the ER lumen in an unfolded state and require protein chaperones and catalysts of protein folding to attain their final appropriate conformation. A sensitive system termed the unfolded protein response (UPR) prevents misfolded proteins from progressing through the secretory pathway and directs them toward a degradative pathway. Proteins such as BiP (a 78-kDa glucose-regulated protein also known as GRP78) and its downstream effector proteins (e.g., PERK, IRE1, and ATF6) have been studied extensively to determine whether ER stress is involved in pathogenesis of diabetes, atheroscle-

rosis, and neurodegenerative disorders. ER stress is implicated in the pathogenesis of diabetic retinopathy and other retinal diseases [48].

We investigated whether chronic stress *in vitro* (oxidative stress model) and *in vivo* stress (diabetes) altered Sigma1R and BiP expression in retinal neurons, how the interaction between these proteins may be altered during oxidative stress, and whether (+)-PTZ alters Sigma1R–BiP binding and expression of ER stress-related genes in these models [20]. We conducted experiments in primary ganglion cells, which showed marked sensitivity to oxidative stress, characterized by neurite process disruption and cellular apoptosis. Oxidative stress increased expression of the proteins that initiate and execute apoptosis (caspase-9 and -3, respectively) and the upstream pro-apoptotic genes *FasL* and*TRAIL*. (+)-PTZ treatment reduced caspase-9 and -3 levels and the pro-apoptotic genes. Expression of the anti-apoptotic gene *Survivin* increased when oxidatively stressed cells were treated with (+)-PTZ. Neurite disruption detected in primary ganglion cells exposed to oxidative stress was not observed in (+)-PTZ-treated cells. We used the RGC5 cell line to analyze the interaction of Sigma1R with BiP under oxidative stress. Exposing these cells to xanthine:xanthine oxidase as an oxidative stressor did not alter Sigma1R protein levels over the 18-hour period examined; however it increased binding of Sigma1R to BiP [20]. When the cells were treated with (+)-PTZ [3 μM], Sigma1R–BiP binding was at baseline level. Our data are similar to those in the glucose-deprivation model [46], wherein Sigma1R–BiP interaction appeared to increase rather than the thapsigargin stress model in which σR1 dissociated from BiP.

Many proteins are regulated by phosphorylation resulting in an increase or decrease of biological activity, movement between subcellular compartments, and interactions with other proteins. We asked whether there are differences in Sigma1R phosphorylation under oxidative stress conditions, specifically phosphorylation of serine and tyrosine residues [20]. While there was no difference in tyrosine phosphorylation under stress, there was a robust increase in phosphorylation of serine (sixfold by 18 h of oxidative stress). Sigma1R serine phosphorylation in oxidatively-stressed cells decreased markedly when the cells were treated with (+)-PTZ. Our studies showed Sigma1R phosphorylation is altered by cellular stress and by ligand treatment. Phosphorylation of Sigma1R may facilitate its binding to BiP, as the increase in Sigma1R–BiP interaction parallels phosphorylation of Sigma1R. Additional studies are needed to demonstrate this potentially interesting and important phenomenon unequivocally. Sigma1R binding to proteins is not limited to BiP; it interacts also with IP_3R_3 receptors [46]. There have been reports that Sigma1R interacts with L-type calcium channels in ganglion cells [19, 49], although reports from del Pozo's group in fura-2-loaded synaptosomes harvested from WT and *Sigma1R*$^{-/-}$ mice suggest that Sigma1R is not involved in calcium influx via calcium channels [50]. Clearly, this is an area that warrants further investigation. It is noteworthy that studies have shown that Sigma1R may be involved in the regulation of output signaling of ganglion cells by preferentially modulating NMDA receptor-mediated light-evoked excitatory postsynaptic currents (eEPSCs) of these retinal neurons [51]. This group showed that suppression of NMDA responses of rat retinal ganglion cells caused by the activation of Sigma1R may be mediated by a distinct intracellular calcium-dependent PLC-PKC pathway [52].

We investigated ER stress in neural retinas from diabetic *Ins2*$^{Akita/+}$ mice treated with (+)-PTZ over the course of several weeks [20]. Several of the same genes that had increased in our *in vitro* system (*BiP*, *PERK*, *IRE1a*, and *ATF4*) were increased in the *in vivo* diabetic model and expression levels were similar to control values when the mice were treated with (+)-PTZ. It appears that as with the *in vitro* system, (+)-PTZ attenuates upregulation of ER stress genes in an *in vivo* model of diabetic retinopathy.

While the role of Sigma1R in ER stress was supported by our data [20], we were interested in other genes (not necessarily directly linked to ER stress) whose expression might be altered by σR1

ligands *in vivo*. We analyzed the retinal transcriptome in diabetic mice using gene array technology [20]. Interesting data emerged showing alterations in diabetic conditions that were reversed with the 4-week (+)-PTZ treatment. Included among the affected genes were *Frzp* and slit homolog 1, genes involved in cell differentiation and axon guidance, respectively. Expression of crystallins γ-B and -D was reversed markedly when diabetic mice were treated with (+)-PTZ. These data are noteworthy, given that proteins of the crystallin superfamily increase dramatically in early diabetic retinopathy reviewed in [53]. Another gene with altered expression in the *Ins2* $^{Akita/+}$ mouse, which was reversed by (+)-PTZ treatment, was VEGF receptor 1. VEGF (vascular endothelial growth factor) is a molecule involved in numerous physiological functions, including angiogenesis. VEGF bioactivity is transmitted through the binding of specific receptors (VEGF receptor 1, 2, and 3). Our data show an elevation of VEGFR1 in diabetic mice compared with WT mice, but a decrease in receptor expression when (+)-PTZ was administered to diabetic animals. A number of other genes related to antioxidant function, axon guidance and calcium signaling were also altered in this model following treatment with (+)-PTZ [20].

18.8 Evaluation of the Eye and Retina of *Sigma1R*$^{-/-}$ Mice

Given its abundant expression in the eye, its role in neuroprotection and cell survival, and its putative molecular chaperone role, we postulated that Sigma1R would be critical for ocular development and/or maintenance of normal ocular structure/function. The consequences of absence of Sigma1R on ocular phenotype had not been investigated, but the availability of genetically manipulated mice lacking *Sigma1R* (*Sigma1R*$^{-/-}$ mice) offered a tool to clarify the role of σR1 in ocular development and disease. In collaboration with Dr. E. Zorrilla, Scripps Institute, La Jolla, CA we established a colony of *Sigma1R*$^{-/-}$ mice. To determine whether Sigma1R was critical for ocular development and/or maintenance of normal ocular structure/function, we used functional, morphologic, and cell biological tools to examine comprehensively the ocular phenotype in *Sigma1R*$^{-/-}$ versus wildtype (WT, *Sigma1R*$^{+/+}$) mice over a 1-year period [54]. The anterior segment of the eye (cornea, lens, and ciliary body-iris) is normal in *Sigma1R*$^{-/-}$ mice and intraocular pressure (IOP) is within normal limits at least through 1 year. In the retina, however, there are electrophysiological changes in *Sigma1R*$^{-/-}$ mice including significantly decreased ERG b-wave amplitudes and diminished negative scotopic threshold responses (nSTR) detected at 12 months, consistent with inner retina dysfunction (Fig. 18.5a). Comprehensive morphometric analyses reveal significantly fewer cells in the ganglion cell layer (GCL) by one year and an increase in cells undergoing apoptosis in this layer (Fig. 18.5b, d). Interestingly, we did not observe dying cells in the GCL in mice that were younger than 6 months; however, we did observe alterations in the optic nerve head (ONH) of the *Sigma1R*$^{-/-}$ mice. At 18 weeks, TUNEL-positive cells were present in the astrocyte-rich region of the ONH, which forms a mesh-like network of glial cells through which ganglion cell axons pass. The astrocytes are intimately associated with axons of the ONH. Within this glial laminar region of the optic nerve, many TUNEL-positive glial cells were detected. The ultrastructural analysis of the ONH revealed disruption of the axonal processes. The axon fibers in the *Sigma1R*$^{-/-}$ mice were swollen with accumulation of various organelles, especially mitochondria. It appears that alterations of the ONH presage the retinal dysfunction and death observed in later months in these mice. The data suggest that σR1 is critical in maintaining inner retinal function [54] The alterations of the ONH and subsequent ganglion cell loss were not accompanied by an increase in IOP or a change in retinal vascularization [54].

Recently, investigations from Guo's lab analyzed whether Sigma1R had any role in an acute retinal injury model [55]. They performed intraorbital optic nerve crush in *Sigma1R*$^{-/-}$ mice and determined that the number of surviving cells in

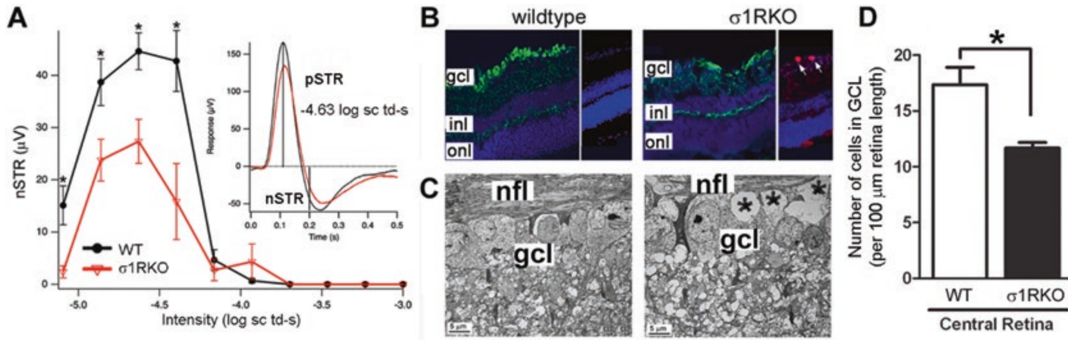

Fig. 18.5 *Late onset retinal dysfunction in Sigma1R*$^{-/-}$ *mice.* Sigma1R is not required for normal retinal development. By 6 months however, dying cells are detected in the optic nerve head; by 1 year there is loss of RGC function (e.g. decreased negative scotopic threshold responses (nSTR) of the ERG (**a**). Retinal cryosections were stained to detect neurons in the ganglion cell layer (gcl) (green fluorescence, *left panels*) or active caspase-3, an apoptosis marker (red fluorescence, *right panels*). There are fewer cells in the gcl of *Sigma1R*$^{-/-}$ mice compared to WT (**b**). Electron microscopy was performed to evaluate health and integrity of ganglion cells in retinas of WT and *Sigma1R*$^{-/-}$ *mice*. Electron micrographs show the nerve fiber layer (nfl) is labeled and just below it are plump RGC bodies in WT retinas. In *Sigma1R*$^{-/-}$ retinas are areas of cell drop out ("*" denotes missing cells) (**c**). The numbers of cells in the ganglion cell layer was quantified and cell loss was worse in the central retina (**d**; *p < 0.05). The complete assessment of this late-onset retinal degeneration has been published [54] (*IOVS*, with permission, portions of figs. 4 and 9, Ref. [54])

the GCL of *Sigma1R*$^{-/-}$ mice was significantly decreased (18.5 %) compared to WT mice subjected to the same injury. Their data strongly support the notion that lack of Sigma1R increases susceptibility to acute retinal injury and they found that if Sigma1R is present in retina crush-induced degeneration, ganglion cell loss is attenuated. The effects of chronic stress to the retina in the absence of Sigma1R, however, had not been explored. We investigated whether the late-onset RGC death reported for *Sigma1R*$^{-/-}$ mice [54] would be accelerated under the chronic stress of diabetes. Diabetes was induced in WT mice and *Sigma1R*$^{-/-}$ mice by injecting streptozotocin at 3 weeks of age [56]. Eyes were evaluated 12 weeks post onset of diabetes when mice were 15 weeks of age. When *Sigma1R*$^{-/-}$ mice non-DB mice were analyzed at this age, no functional deficits or structural alterations were observed, confirming earlier findings [54]. However, rendering the *Sigma1R*$^{-/-}$ mice diabetic accelerated retinal dysfunction [56]. Retinas were examined functionally by assessing IOP and nSTRs. *Sigma1R*$^{-/-}$-DB mice had IOPs that were significantly elevated at night compared to *Sigma1R*$^{-/-}$ non-DB mice as well as to WT non-DB and WT-DB mice. The levels detected were ~15 mmHg, which is within the normal range; nevertheless, the elevation in *Sigma1R*$^{-/-}$-DB was significantly greater than in the other mouse groups examined. We also performed functional tests on the animals and detected a marked decrease in nSTRs in the *Sigma1R*$^{-/-}$-DB mice compared to the other mice in the study. The nSTRs ranged between 9 and 13 μV for WT non-DB, WT-DB, and *Sigma1R*$^{-/-}$-non-DB mice compared to ~5 μV in the *Sigma1R*$^{-/-}$-DB mice [56]. The nSTR is a highly sensitive test for RGC activity; thus, these data provide strong evidence that σR1 modulates ganglion cell function under chronic stress. As was the case with acute stress [55], chronic stress can accelerate ganglion cell dysfunction in the absence of Sigma1R. Accompanying the decreased ganglion cell function was a decrease in the numbers of Brn3a-positive cells detected in the ganglion cell layer of *Sigma1R*$^{-/-}$-DB mice compared with WT mice. Our data clearly show that there is a much earlier loss of ganglion cells and evidence of inner retinal dysfunction in *Sigma1R*$^{-/-}$-DB mice compared with WT mice; this supports the role of Sigma1R in forestalling retinal stress. There was a decrease in the number of ganglion cells in *Sigma1R*$^{-/-}$-DB compared to *Sigma1R*$^{-/-}$-(nondiabetic), although the decrease

was similar to the decrease in cell number between WT and WT-DB. It appears that cell loss as an endpoint is not as severe an indicator as the nSTR and IOP changes we observed [56]. The *in vivo* data comparing diabetic versus nondiabetic *Sigma1R*$^{-/-}$ mice allowed us to investigate the role of chronic stress on retinal function in the absence of Sigma1R. The acceleration of ganglion cell dysfunction during chronic diabetic stress coupled with the late onset inner retinal dysfunction of nondiabetic *Sigma1R*$^{-/-}$ mice underscores the role of this protein as a stress modulator.

18.9 Evidence that Sigma1R Is Required for (+)-PTZ Retinal Neuroprotection

In *in vitro* and *in vivo* studies, we observed considerable neuroprotection using (+)-PTZ. We took advantage of the observation that the absence of Sigma1R does not hinder normal retinal development [54] and used *Sigma1R*$^{-/-}$ mice to investigate whether the previously reported neuroprotective effects of (+)-PTZ are mediated via Sigma1R. While (+)-PTZ is considered a highly specific ligand for Sigma1R with an affinity in the nanomolar range (0.0046 μM [K_d]) [57], it had not been demonstrated unequivocally that (+)-PTZ mediates neuroprotection via Sigma1R. Moreover, there have been reports of alternative targets for (+)-PTZ [58]. Thus, we designed an experiment to examine this question specifically in ganglion cells, because of their vulnerability in diabetic retinopathy [29]. By isolating ganglion cells from WT mice and from *Sigma1R*$^{-/-}$ mice, we had a neuronal population that either did or did not contain Sigma1R. The cells could be manipulated using a known stressor and we had clear endpoints (neurite processes, TUNEL positivity) that could be analyzed in a straightforward manner to inform whether (+)-PTZ afforded protection. RGCs were isolated following a well-established immunopanning procedure from neonatal WT and *Sigma1R*$^{-/-}$ mice; they were cultured under oxidative stress conditions with or without (+)-PTZ. The data showed that ganglion cells from *Sigma1R*$^{-/-}$ mice succumbed to oxidative stress in a manner similar to those harvested from WT; however, unlike WT-treated cells, (+)-PTZ did not prevent death in cells isolated from *Sigma1R*$^{-/-}$ mice. That is, (+)-PTZ did not protect against oxidative stress in cells lacking Sigma1R. These findings provide compelling evidence that neuroprotective effects of (+)-PTZ are obligatorily dependent on Sigma1R. Our recent studies demonstrating cone photoreceptor preservation in *rd10* mice revealed no protective effects if (+)-PTZ was administered in *rd10/Sigma1R*$^{-/-}$ mice [43].

18.10 Additional Mechanisms of Sigma1R Neuroprotection Involving Retinal Müller Glial Cells

Several laboratories have been exploring mechanisms by which Sigma1R mediates retinal neuroprotection and many of these investigators describe their findings in other chapters in this book. Our group has been exploring how Sigma1R may mediate neuronal protection via its actions on glial cells, particularly Müller glial cells. The interactions between glia and neurons contribute to retinal homeostasis as reviewed in detail [59]. It has been proposed that cooperativity exists among retinal cells that arise from a common stem cell to form a columnar array [60]. The idea is that the retina is constituted by many functional units in which local interactions occur between the group of retinal neurons and their supportive Müller glial cell, limiting the sphere of influence of the latter. Thus, each Müller cell may only have to meet the requirements of its immediate neighbors for the extracellular environment to remain stable in the face of intense neural activity.

We had established earlier that Sigma1R is present in Müller cells [36] and we knew that Müller cells isolated from *Sigma1R*$^{-/-}$ mice showed an increase in ER stress proteins [61]. A common feature of retinal disease is Müller cell reactive gliosis, which includes cytokine release. We investigated whether lipopolysaccharide

(LPS) stimulates cytokine release by primary mouse Müller cells and whether (+)-PTZ could alter that release [62]. Using a highly sensitive inflammatory cytokine array we observed significant release of macrophage inflammatory proteins (MIP1γ, MIP2, MIP3α) and interleukin-12 (IL12 (p40/p70)) in LPS-treated cells compared to controls, and a significant decrease in secretion upon (+)-PTZ treatment. Müller cells from *Sigma1R*$^{-/-}$ mice demonstrated increased MIP1γ, MIP2, MIP3α and IL12 (p40/p70) secretion when exposed to LPS compared to LPS-stimulated WT cells. Cells exposed to LPS demonstrated increased NFκB nuclear location, which was reduced significantly by (+)-PTZ-treatment. NFκB, which is the abbreviation for nuclear factor kappa-light-chain-enhancer of activated B cells, is a protein complex that controls transcription of DNA, cytokine production and cell survival. Media conditioned by LPS-stimulated-Müller cells induced leukocyte-endothelial cell adhesion and endothelial cell migration, which was attenuated by (+)-PTZ treatment [62]. The findings suggest that release of certain inflammatory cytokines by Müller cells can be attenuated by Sigma1R ligands providing insights into the retinal neuroprotective role of this receptor.

Reactive gliosis can also be caused by oxidative stress, which figures prominently in retinal diseases, including diabetic retinopathy, glaucoma, and retinitis pigmentosa. Since Müller cells are essential for homeostatic support of the retina, we investigated whether Sigma1R mediates the oxidative stress response of Müller cells using WT and *Sigma1R*$^{-/-}$ mice [63]. We observed increased endogenous reactive oxygen species (ROS) levels in *Sigma1R*$^{-/-}$ mouse Müller cells compared to WT, which was accompanied by decreased expression of the genes encoding antioxidants Sod1, catalase, Nqo1, Hmox1, Gstm6, and Gpx1. The protein levels of SOD1, CAT, NQO1, and GPX1 were also significantly decreased. The genes encoding these antioxidants contain an antioxidant response element (ARE), which under stress is activated by NRF2, a transcription factor that typically resides in the cytoplasm bound by KEAP1. In the *Sigma1R*$^{-/-}$ Müller cells, Nrf2 expression was decreased significantly at the gene (and protein) level, whereas Keap1 gene (and protein) levels were markedly increased. NRF2-ARE binding affinity was decreased markedly in *Sigma1R*$^{-/-}$ Müller cells. We investigated system xc(−), the cystine-glutamate exchanger, which is critical for synthesis of glutathione (GSH) [64], and observed decreased function in *Sigma1R*$^{-/-}$ Müller cells compared to WT as well as decreased GSH and GSH/GSSG ratios [63]. This was accompanied by decreased gene and protein levels of xCT, the unique component of system xc(−). Thus it appears that Müller glial cells lacking Sigma1R manifest elevated ROS, perturbation of antioxidant balance, suppression of NRF2 signaling, and impaired function of system xc(−). The data suggest that the oxidative stress-mediating function of retinal Müller glial cells may be compromised in the absence of Sigma1R. The neuroprotective role of Sigma1R may be linked directly to the oxidative stress-mediating properties of supportive glial cells. Future studies to evaluate the role of Sigma1R in modulating NRF2 and KEAP1 will provide important insights about whether this constitutes a key mechanism by which Sigma1R mediates retinal neuroprotection.

18.11 A Note About Sigma2R

Sigma receptor 2 is much less studied than Sigma1R, and is thought to be a distinct protein that shares the ability to bind some ligands common to both receptors. Whether the two receptors share overlapping biological functions is unknown. Recently, progesterone receptor membrane component 1(PGRMC1) was shown to contain the putative Sigma2R binding site [66], although additional studies suggest otherwise indicating that this issue is controversial [67]. PGRMC1 has not been studied in retina. We hypothesized that biological interactions between Sigma1R and PGRMC1 would be evidenced by compensatory upregulation of PGRMC1 in *Sigma1R*$^{-/-}$ mice. Immunofluorescence, RT-PCR, and immunoblotting methods were used to analyze expression of

PGRMC1 in wild-type mouse retina and tissues from *Sigma1R*$^{-/-}$ mice were used to investigate whether a biological interaction exists between Sigma1R and PGRMC1 [67]. We found that in the eye, PGRMC1 is expressed in corneal epithelium, lens, ciliary body epithelium, and retina. In retina, PGRMC1 is present in Müller cells and retinal pigment epithelium. This expression pattern is similar, but not identical to Sigma1R. PGRMC1 protein levels in neural retina and eye cup from *Sigma1R*$^{-/-}$ mice did not differ from WT mice. Nonocular tissues, lung, heart, and kidney showed similar Pgrmc1 gene expression in WT and *Sigma1R*$^{-/-}$ mice. In contrast, liver, brain, and intestine showed increased *Pgrmc1* gene expression in *Sigma1R*$^{-/-}$ mice. If indeed Sigma2R is PGRMC1 [66], our work showed that deletion of Sigma1R did not result in compensatory change in PGRMC1 [67]. Future studies await the precise clarification of the molecular identity of Sigma2R at which time its role in retina can be investigated comprehensively.

18.12 Conclusions

Sigma receptors were first described 40 years ago and since that time there have been nearly 4000 articles published regarding the function of this enigmatic protein. Many of the studies have focused on its role in brain and in neurodegenerative diseases. Over the past 20 years, there have been increasing reports of the role of Sigma1R in the eye. Within the eye, the light sensitive retina has been the subject of many studies. As noted in this chapter and several others in this book, a number of very important roles have been ascribed to Sigma1R. In this chapter we have attempted to provide a brief overview of the earliest pharmacological studies showing that Sigma1R was present in eye followed by descriptions of studies localizing Sigma1R in various tissues including retina. Increasingly, it has become clear that Sigma1R ligands exert profound neuroprotection in retina including in retinal ganglion cells and now more recently in photoreceptor cells. Studies have utilized cell lines and primary cell culture to determine mechanisms by which Sigma1R effects neuroprotection. The availability of mice lacking Sigma1R (*Sigma1R*$^{-/-}$ mice) has provided a powerful tool to explore the role of Sigma1R in retina and to evaluate the specificity of Sigma1R ligands. Data suggest that in retina, Sigma1R plays a key role in modulating cellular stress.

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19 Peeking into Sigma-1 Receptor Functions Through the Retina

Timur A. Mavlyutov and Lian-Wang Guo

Abstract

This review discusses recent advances towards understanding the sigma-1 receptor (S1R) as an endogenous neuro-protective mechanism in the retina, a favorable experimental model system. The exquisite architecture of the mammalian retina features layered and intricately wired neurons supported by non-neuronal cells. Ganglion neurons, photoreceptors, as well as the retinal pigment epithelium, are susceptible to degeneration that leads to major retinal diseases such as glaucoma, diabetic retinopathy, and age-related macular degeneration (AMD), and ultimately, blindness. The S1R protein is found essentially in every retinal cell type, with high abundance in the ganglion cell layer. Ultrastructural studies of photoreceptors, bipolar cells, and ganglion cells show a predominant localization of S1R in the nuclear envelope. A protective role of S1R for ganglion and photoreceptor cells is supported by in vitro and in vivo experiments. Most recently, studies suggest that S1R may also protect retinal neurons via its activities in Müller glia and microglia. The S1R functions in the retina may be attributed to a reduction of excitotoxicity, oxidative stress, ER stress response, or inflammation. S1R knockout mice are being used to delineate the S1R-specific effects. In summary, while significant progress has been made towards the objective of establishing a S1R-targeted paradigm for retinal neuro-protection, critical questions remain. In particular, context-dependent effects and potential side effects of interventions targeting S1R need to be studied in more diverse and more clinically relevant animal models.

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Keywords

Sigma-1 receptor • Retinal diseases • Retinal ganglion neurons • Müller glia • Photoreceptors • Neuro-protection

19.1 Introduction

Earlier pharmacological profiling revealed two subtypes of sigma receptors (S1R and S2R) [1]. The S1R sequence has been cloned [2], while the identity of S2R remains unknown [3–5]. Despite numerous studies since its discovery [6, 7], S1R remains mysterious. Outstanding questions include the following: (1) *Identity*. No homolog of S1R is found in mammalian genomes. Curiously, the only protein that shares >30 % sequence identity with S1R is the yeast C-8,7 sterol isomerase [8]. But S1R is not found in yeast and it does not possess sterol isomerase activity. While an NMR structure of partial S1R was recently reported [9], an atomic structure of the whole protein has yet to be unveiled. (2) *Function*. In contrast to its unique identity, S1R is ubiquitously distributed, with high abundance in the central nervous system and liver [8]. Paradoxically, while S1R knockout mice do not exhibit overt phenotypes [10], S1R is linked to an array of pathological conditions such as cancer and neurological disorders (see review) [11]. These studies were conducted mostly using S1R ligands with only a handful employing S1R knockout mice. Hence, the S1R specificity of observed ligand functions awaits further investigations in knockout animals. (3) *Endogenous ligands*. Many synthetic ligands bind to S1R, including a few that have been intensively used for investigating S1R functions (see review) [12]. However, the identity of the true endogenous S1R ligand remains unclear. Several naturally occurring compounds show affinity for S1R, including steroids [13], trace amine [14], and lipids [15–17], but their S1R-specific roles are largely unknown.

Recently, there has been a surge of interest in S1R. In particular, important progress has been made to unravel its important role in the nervous system. A potential neuro-protective function of S1R is found in animal models of major neurodegenerative diseases including Alzheimer's disease [18], Parkinson's disease [19], amyotrophic lateral sclerosis [20, 21], as well as retinal degenerative diseases [22–25]. The retina presents an excellent model system for studying S1R functions in the central nervous system. The main advantages include the following: (1) The retina is integral to, yet isolated from the brain, thus conveniently accessible for experimentation. (2) Animal models are available for major retinal degenerative diseases. (3) Despite being a thin sheet of tissue, the retina contains diverse cell types including neurons, epithelial cells, macroglia and microglia (see review) [26]. (4) Retinal cells are exquisitely organized into distinct layers [26], and hence advantageous for morphological and pathophysiological investigations (for example, see Fig. 19.1). (5) Since the eye is an immunologically privileged organ [27], immunogenic concerns caused by introducing experimental or therapeutic agents are relatively minor. In spite of a limited number of publications on S1R in the retina, progress has been achieved in identifying neuro-protective functions of S1R. While excellent reviews are available for studies of S1R in the nervous system in general [8, 11, 28], an overview is lacking for studies on S1R specifically in the retina. Here we discuss recent findings on the distribution, function, and molecular mechanisms of S1R in the mammalian retina.

19.2 General Molecular Functions of S1R

Mammalian S1R is a protein of 223 amino acids, with two transmembrane helices and a hydrophobic C-terminal region that putatively form a ligand binding pocket(s) (see review) [12]. An N-terminal double-arginine sequence serves as an endoplasmic reticulum (ER) retention motif.

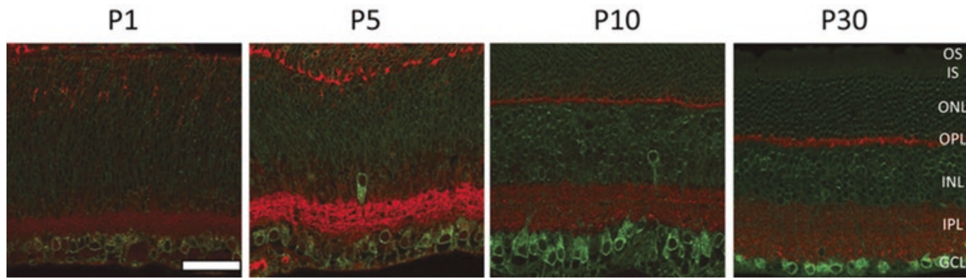

Fig. 19.1 Immunostaining of S1R on mouse retinal sections at different postnatal stages. *Green*, S1R; *red*, synaptophysin. *OS* outer segment, *IS* inner segment, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer. Scale = 50 µm (Adapted from: Scientific Reports. 2015.2;5:10689)

Until the discovery of its ligand-operated chaperone function [29], molecular functions of S1R were not known. This study revealed that activated S1R regulates mitochondrial calcium homeostasis by stabilizing the IP3 type 3 receptor at ER/mitochondria contacts. Thus S1R is thought to support cell survival [29]. Follow-up studies suggest that S1R is a multitasking protein involved in a broad range of cellular activities. S1R has been reported to modulate the activity of various, e.g., Na^+, K^+, Ca^{2+}, Cl^-, ion channels, likely via direct interactions [30] (see review) [28]. S1R was also reported to interact with G-protein coupled receptors (see review) [12]. Recently, evidence showed that S1R is involved in autophagy [31, 32]. In accordance, S1R also participates in ER stress responses; e.g., S1R interacts with and stabilizes ER stress sensor IRE1 [29, 33, 34]. While interactions of S1R with several lipids were observed earlier (see review) [12], most recently the Su group reported that S1R transports myristic acid to support proper tau phosphorylation and axon extension [17]. They also found that S1R modulates transcriptional activities via interaction with a nuclear envelope protein [35], consistent with the presence of S1R in the nuclear envelope [36, 37].

Despite continuous discoveries about the molecular biology of S1R, it remains an open question as to whether there is a common thread connecting S1R functions. In other words, can all S1R actions be attributed to its chaperone activity? The S1R C-terminal half is believed to be responsible for its chaperone activity [29, 38]. If the C-terminus of S1R is confined in the ER lumen, how would S1R functionally interact with cytosolic proteins? What are the functions of the other S1R domains, for instance, the central loop region proposed to be cytosolic? It was reported that ligand binding to S1R alters its monomeric/oligomeric states [39–41]. How is this functionally related? In sum, many intriguing questions remain, which would inspire new investigations to help understand S1R functions in the retina as well as other systems.

19.3 Cellular and Sub-cellular Distribution of S1R in the Retina

The neural retina is a sheet of light-sensitive tissue in the back of the eye. Its intricate structure contains three layers of neatly organized neurons: the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL). Sandwiched in between are two synaptic layers connecting neurons: the outer plexiform layer (OPL) and the inner plexiform layer (IPL) [26] (Fig. 19.1). The neural retina rests on a nourishing single layer of pigmented cells called retinal pigment epithelium (RPE). ONL is formed by photoreceptors. INL contains bipolar cells, horizontal cells, and amacrine cells. GCL is mainly composed of ganglion cells and displaced amacrine cells. Müller glia cells traverse the entire neural retina and are interconnected with retinal

microglia cells [42]. Vision begins at photoreceptors, which convert light signals into chemical signals and then electrical impulses. Filtered through secondary neurons in INL, the electrical signals are transmitted to ganglion cells, and sent further into the brain through their axons (optic nerve) to be processed into images or other forms of vision.

While differences may exist among species, S1R is found in all cellular layers in the mouse retina, including GCL, INL, ONL, and RPE, as detected by both in-situ hybridization and immunohistochemistry [23, 37, 43, 44]. The specificity of S1R immunostaining is confirmed by the lack of S1R-positive staining in the retina of S1R knockout mouse [23]. S1R is abundant in GCL in mouse [23, 43], rat [45], monkey, pig, and human retinas [37]. In contrast, staining of S1R is less intense in ONL, unclear in the photoreceptor inner segment, and not detectable in the outer segment [23]. Consistently, using immunoelectron microscopy (EM) we did not observe S1R-positive staining on outer segment membrane discs, or in mitochondria or the ER which are concentrated in the photoreceptor inner segment [37] (Fig. 19.2). Rather, S1R is exclusively localized in photoreceptor nuclear membranes. Similarly in bipolar cells, EM data show that S1R is predominantly present in the nuclear envelope, in both outer and inner nuclear membranes [37]. In ganglion cells, S1R is found not only in nuclear membranes, but also in the ER and lipid droplets (Fig. 19.3). Interestingly, in accordance with S1R localization in nuclear membranes in retinal neurons [37] and Müller cells [36], Tsai et al. reported that S1R influences gene transcription by interacting with the nuclear envelope protein emerin to recruit chromatin-remodeling proteins [35].

In spite of new findings, perplexing questions remain about the distribution of S1R in the retinal neurons. For example, S1R was identified as a molecular chaperone functioning at the ER/mitochondria junction [29]. What is the function of S1R in photoreceptor cells, where it is found neither in the ER or mitochondria? S1R has been reported to interact with multiple ion channels including NMDA receptors in the ganglion cell plasma membrane [46, 47]. However, EM data do not show S1R in the plasma membrane of retinal neurons [37]. Is it possible some channels in the plasma membrane interact with S1R localized in the subsurface ER cisternae [20, 37] ? Moreover, S1R expression in the embryonic (E16) mouse retina is barely detectable but continuously increases during development until a mature retina is formed [37]. Is there a possible link between the temporal S1R distribution and retinal development?

19.4 Functions of S1R in the Retina

High-affinity S1R-selective agonists, e.g., (+)-pentazocine, PRE084, SK10047, and antagonists, e.g., NE100, BD1047, BD1063, present convenient pharmacological tools for studying S1R functions in the retina [12]. Using S1R ligands to treat animals (or cells) and whole retina samples for analysis, early studies suggested a neuro-protective role of S1R in the retina [43, 48–52]. Taking advantage of the layered retinal structure that partitions different neurons, in recent studies investigators analyzed cell type-specific S1R functions in the retina (see details in the subsections below). Moreover, S1R knockout mice [10] and retinal disease models provide powerful genetic tools for delineating S1R-specific functions in a given disease or cell type. However, mechanistic studies using isolated retinal neurons, in particular, photoreceptor cells, are challenging, as these highly specialized neurons cannot maintain their physiology and viability in cell culture. While immortalized cell lines are often used to represent corresponding retinal neurons, they are very dissimilar to mature native neurons in morphology and pathophysiology. Moreover, they may be associated with identity complications, e.g., the RGC-5 cell line [53]. Likely because of available methods to culture primary ganglion cells and their high S1R abundance [37, 43, 44], a majority of S1R functional studies in the retina have focused on ganglion neurons and associated disease conditions. Studies have also been extended to other cell types, e.g., Müller glia and microglia. In the following subsections, published studies on each retinal cell type will be discussed.

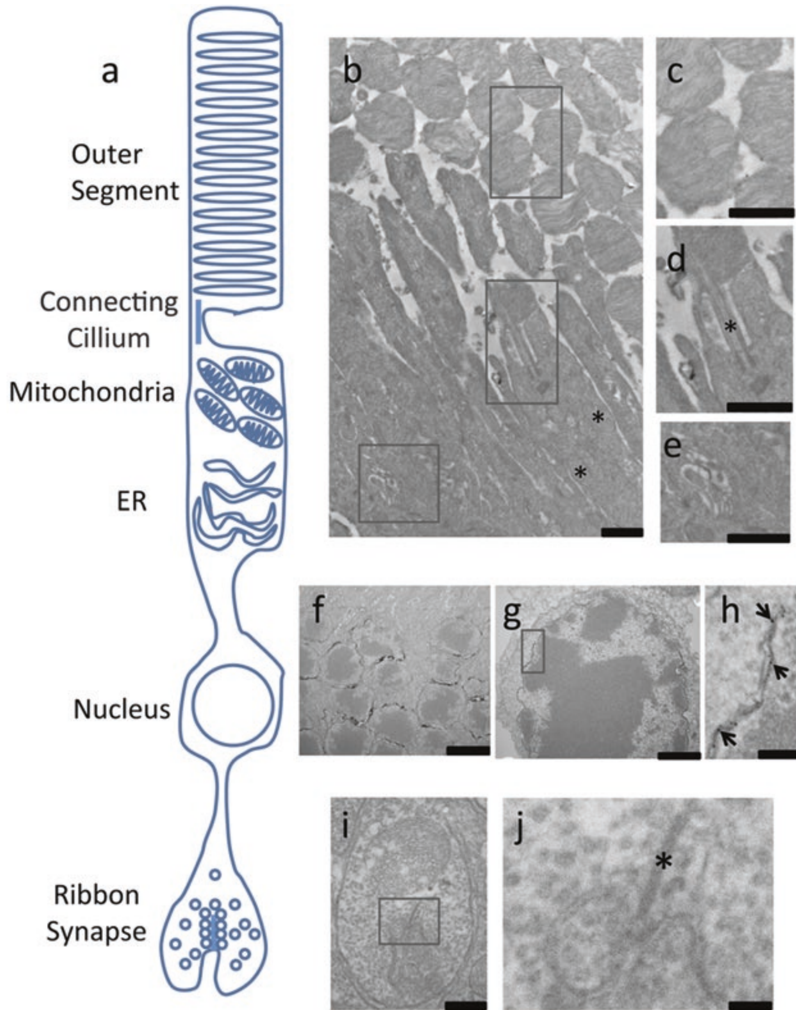

Fig. 19.2 Electron microscopy images showing S1R distribution in the mouse photoreceptor subcellular compartments. (**a**) Schematic of the compartments in the photoreceptor. (**b**) Ultrastructure of outer and inner segment. *Asterisks* label mitochondria. (**c**–**e**) Magnified images of the *boxed areas* in (**b**), showing the outer segment containing membrane discs, the connecting cilium (asterisk), and the inner segment (including ER), respectively. (**f**–**h**), Localization of S1R in the nuclear envelope. (**f**) nuclear region of several photoreceptor cells; (**g**) nuclear envelope of a single cell, (**h**) magnified *box area* in (**g**) showing S1R localization in the outer and inner membranes of the nuclear envelope (pointed to by *arrows*). (**i**) and (**j**), Photoreceptor synaptic terminal. The image in (**j**) is a magnified box area in (**i**) revealing the characteristic ribbon (*asterisks*) and vesicles. Scales: (**b**–**e**) and (**g**), 1 μm; (**f**), 3 μm; (**h**), 0.2 μm; (**i**), 0.5 μm; (**j**), 0.1 μm (Adapted from: Scientific Reports. 2015.2;5:10689)

19.4.1 Retinal Ganglion Cells (RGCs)

RGCs make the functional link between the retina and the brain. Approximately 20 subtypes of RGCs process complex visual information collected from bipolar cells and amacrine cells, and then send it to the brain as action potentials along RGC axons (see review) [26]. As such, RGCs play a critical role in vision, and their deterioration leads to vision loss or impairment. A good example is glaucoma, a prevalent retinal disease characterized by final-stage RGC loss and consequent visual field deficits (see review) [54]. Although no data is available with regard to S1R expression in each specific RGC subtype, it is conceivable that S1R is ubiquitously expressed, based on S1R-positive staining in essentially all GCL cells [23, 37, 43].

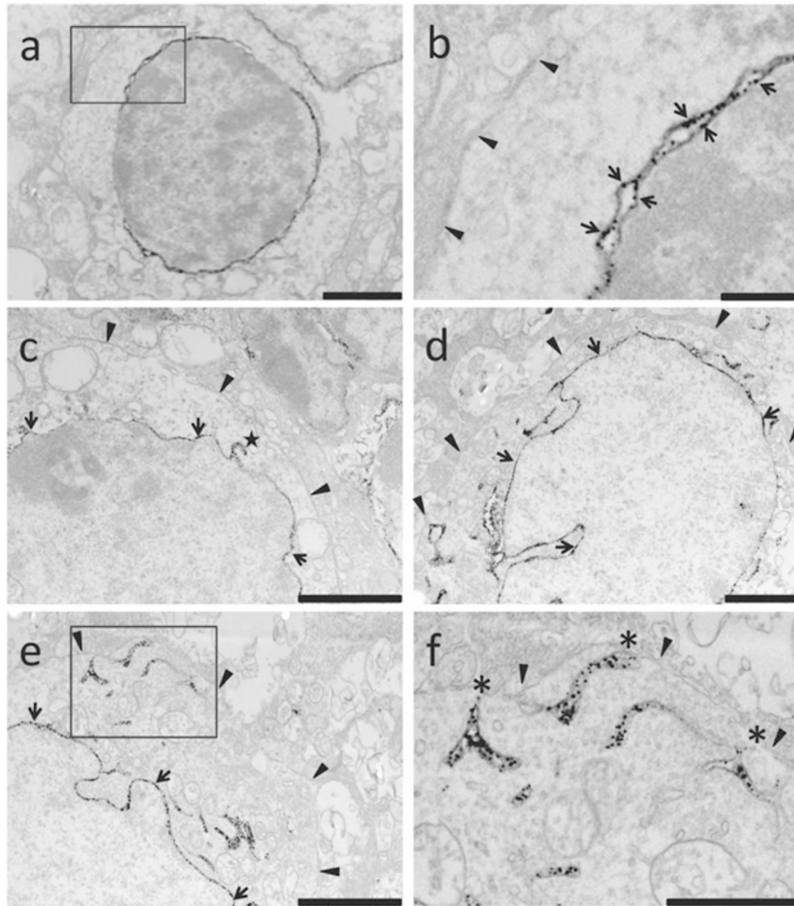

Fig. 19.3 Subcellular localization of S1R in bipolar and ganglion cells of the mouse retina. (**a**–**c**), Bipolar cells. (**b**) shows magnification of the *boxed area* in (**a**). *Arrows* point to S1R immunolabeling in the inner and outer membranes of the nuclear envelope. *Arrowheads* mark the plasma membrane. (**c**) shows S1R localization in the ER membrane (*star*) connected to the nuclear envelope (*arrows*). (**d**–**f**), Ganglion cells. (**d**) shows predominant S1R localization in the nuclear envelope (*arrows*) but not in the plasma membrane (*arrow heads*). (**e**) highlights the presence of S1R in the ER (*boxed area*). (**f**) shows the magnification of the boxed area in (**e**), revealing S1R localization in the ER cisternae (*asterisks*) that are adjacent to the plasma membrane (*arrow heads*). Scales: (**a**), (**c**–**e**), 2 μm; (**f**), 1 μm; (**b**), 0.25 μm (Adapted from: Scientific Reports. 2015.2;5:10689)

In vitro and in vivo studies from several research groups support a pro-survival role of S1R in RGCs. Using both primary mouse RGCs and a RGC-5 cell line, the Smith group showed that the S1R-specific agonist (+)-pentazocine protected against apoptosis induced by homocysteine or glutamate. The mechanism was attributed to the attenuation of excitotoxicity, which was mediated by the NMDA receptor [55, 56]. In a recent study, they observed that (+)-pentazocine also protected RGC-5 cells against oxidative stress; this effect was associated with downregulation of ER stress proteins [57]. Using whole-cell patch clamp on RGC-5 cells, the Yorio group found that the S1R agonist SKF10047 promoted cell survival by inhibiting apoptosis-provoking Ca^{2+} influx mediated by the L-type Ca^{2+} channel [58, 59]. In purified rat primary RGCs, they were able to recapitulate the inhibitory effect of S1R activation on Ca^{2+} influx and a possible S1R/L-type channel interaction [60]. Their latest work showed that S1R protected RGCs in vitro against ischemic damage via ERK activation [61]. In an ex vivo study using patch clamp on rat retinal slices, the Yang group observed suppression of NMDA receptor-specific current

responses in both ON and OFF types of RGCs following S1R activation [47]. Their data further suggested that this effect was mediated through a Ca^{2+}-dependent PLC-PKC pathway. In sum, all the foregoing in vitro studies suggest a protective role of activated S1R in RGCs, via attenuation of oxidative stress, excitotoxicity, or Ca^{2+} toxicity involving ion channels. At present it is not clear whether these S1R actions are orchestrated in RGCs under cellular stresses.

In an in vivo study using a spontaneous diabetic retinopathy mouse model, the Smith group identified a prominent anti-oxidative effect of S1R activation [22]. Treating animals with (+)-pentazocine injection preserved the thickness of IPL and INL, cell number in GCL, as well as organization of Müller glia. Demonstrating a specific role of S1R in ganglion cell neuro-protection, the Guo group reported that cell loss in GCL was significantly faster in S1R knockout mice compared to wild type control after optic nerve crush, an acute glaucoma model [23] (Fig. 19.4). This observation was echoed by another study using S1R knockout mice from the Smith group [62].

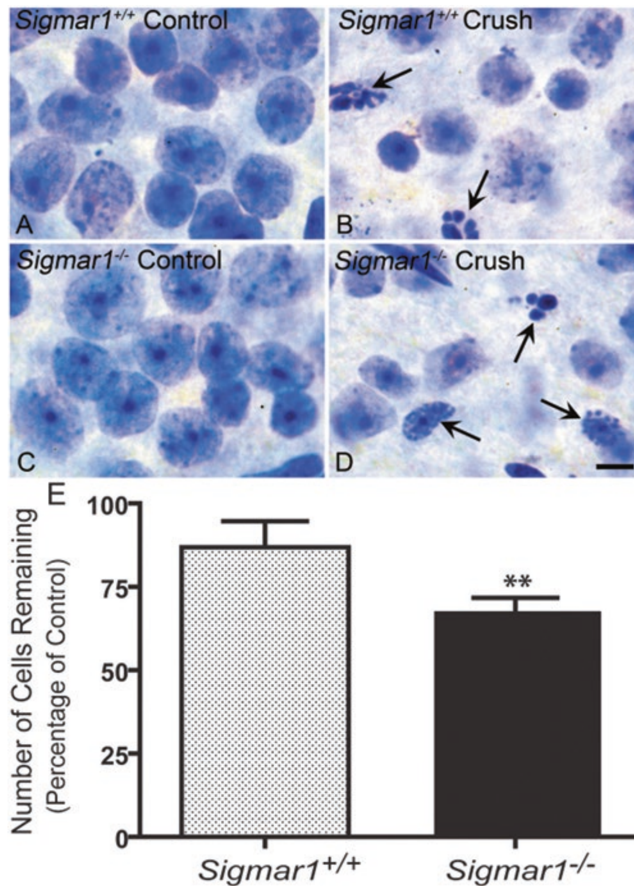

Fig. 19.4 Comparison of the post-crush cell loss in the retinal ganglion cell layer between WT and Sigmar1$^{-/-}$ (S1R knockout) mice. (**a–d**) Nissl-stained retinal whole-mounts from WT (**a** and **b**) and *Sigmar1*$^{-/-}$ (**c** and **d**) mice. Images were from representative fields (1000×) of the mid-peripheral inferior retinas of 12-month-old mice. For each mouse, while the *right eye* served as untreated control (**a** and **c**), the *left eye* was treated by optic nerve crush for 3 s (**b** and **d**). Retinal whole-mounts were prepared 7 days after surgery, and the side of the ganglion cell layer was stained. Healthy ganglion cells exhibited larger somas and nuclei with prominent nucleoli. *Arrows* point to apoptotic cells. (**e**) Quantification of cells remaining in the retinal ganglion cell layer 1 week after surgery. The number of remaining cells in the experimental eye is represented as a percentage of the untreated control. The data were pooled from three WT and *Sigmar1*$^{-/-}$ pairs of 6-month-old mice and two pairs of 12-month-old mice. There were 86.82 ± 7.90 % (mean±standard deviation [SD], n = 5) cells remaining in WT mice and 68.31 ± 3.36 % remaining in *Sigmar1*$^{-/-}$ mice. ** *t*-test, p = 0.0013 (Adapted from Mol Vis. 2011;17:1034–1043)

While retinal morphology and electroretinogram (ERG) appeared normal in younger S1R knockout mice, decrease of ERG b-wave amplitudes and GCL nuclei number, as well as disrupted axon structure in the optic nerve head, occurred in S1R knockout mice compared to wild type at 12 months of age. Moreover, using S1R knockout mice, they confirmed a S1R-specific neuroprotective function of (+)-pentazocine in an induced diabetic mouse model [63]. Taken together, these studies support an important role of S1R in alleviating RGC stress and degeneration in RGC disease models.

Given the complexity of the pathophysiology of retinal neuro-degeneration, it is a daunting challenge to delineate the molecular mechanisms of S1R-specific neuro-protection for RGCs. Since primary, mature RGCs do not divide and hence they cannot be expanded in cell culture, it is difficult to perform in vitro mechanistic studies using these cells. As RGC-5 which was long used as an RGC line recently proved false [53], it is imperative to establish an appropriate RGC line, for in vitro mechanistic research. Moreover, further investigation is needed to better correlate in vitro mechanisms to in vivo pathophysiology. To better understand the therapeutic potential of targeting S1R for interventions, in particular for treating chronic diseases such as glaucoma, more clinically relevant animal models, e.g., DBA/2J [54], may be utilized. To this end, local drug delivery methods integrating advanced bioengineering technologies would provide new insights and opportunities.

19.4.2 Müller Glia and Microglia

RGCs and Müller glia are closely situated, facilitating their functional interactions in RGC pathophysiology (see review) [42]. In a retinal transcriptome survey, Ha et al. did not find significant changes of ER stress genes in neural retinas isolated from S1R knockout mice compared to wild type control. Interestingly, however, marked expression changes of those genes were observed in Müller cells isolated from knockout versus wild type mice [24]. This finding implies an important role of Müller cells in previously observed S1R-mediated protection of RGCs.

Müller cells are a major glial cell type in the retina where they serve as anatomical conduits between neurons and their environment [42]. Müller cells are radially oriented, spanning the entire thickness of the retina from the inner limiting membrane to the outer limiting membrane. Studies suggest that Müller cells play essential roles in the retina (see review) [64]. In addition to supporting the structural integrity of the retina, they maintain retinal homeostasis by participating in essential processes such as glucose metabolism, antioxidant production, ion/substrate exchange, and vascular regulation. Müller cells, together with astrocytes and microglia, become reactive in retinal diseases [27].

In a recent report, the Smith group observed an increase of LPS-stimulated secretion of inflammatory proteins from Müller cells isolated from S1R knockout mice versus those from wild type control [65]. Furthermore, (+)-pentazocine treatment of Müller cells inhibited the secretion of inflammatory proteins and NFκB translocation to the nucleus. In a follow-up study, they found that Müller cells from S1R knockout mice compared to wild type cells manifested more severe oxidative stress, which could be explained by suppressed NRF2 signaling and impaired function of an L-cysteine/L-glutamate antiporter (system xc^-) [66]. These studies uncovered an essential role of S1R in the suppression of oxidative stress and inflammation in retinal Müller glia. Reporting a different S1R action, Vogler et al. showed that PRE084 mitigated osmotic swelling of Müller cell somas induced by superfusion of rat retinal slices with a hypo-osmotic solution [67]. This S1R effect was likely mediated through activation of a glutamatergic-purinergic signaling cascade known to prevent osmotic Müller cell swelling. Astrocytes are another type of retinal glia that are most abundant in the optic nerve head [64]. To our knowledge, there is no report investigating S1R function in this specific cell type in the retina.

Recently, S1R protein was also found in retinal microglia. Pretreatment of isolated microglia with (+)-pentazocine reduced LPS-stimulated morphological change, intracellular ROS pro-

duction, and secretion of inflammatory cytokines (TNF-α, IL-10, MCP-1). The (+)-pentazocine effects were blocked by S1R antagonist BD1063, suggesting a S1R-specific function [68]. These S1R-mediated responses likely involved suppression of the ERK/JNK MAPK pathway due to S1R activation.

Together, the foregoing reports have brought about a new perspective that S1R may protect RGCs through their functions in Müller glia and/or microglia. They also raise an interesting scenario in which the mechanisms of S1R-mediated retinal neuro-protection are multifactorial, likely involving both neuronal and non-neuronal cells and their interactions. An ensuing question is whether retinal glia or microglia cells can serve as effective therapeutic targets. These cells could play opposite roles. Whereas they are essential for maintaining retinal neuron homeostasis [64], when activated by stress conditions, they may transform into inflammatory cells causing harm to retinal neurons. On the other hand, these cells can be readily isolated from the retina, an advantage for in vitro experimental models. Nonetheless, more studies are warranted to understand their role in retinal neuro-degeneration and protection, in the context of specific S1R-associated regulations.

19.4.3 Bipolar Cells, Horizontal Cells, and Amacrine Cells

The nuclei of bipolar, horizontal, and amacrine cells are all in INL, which is situated in between the photoreceptor layer (ONL) and GCL. In mammalian retinas there are approximately a dozen types of bipolar cells, three types of horizontal cells, and 30 types of amacrine cells (see reviews) [26, 54]. Bipolar cells transfer visual signals either directly from photoreceptors to ganglion cells or indirectly through horizontal cells and amacrine cells. Whereas horizontal cells transmit (and modulate) the visual information from photoreceptors to bipolar cells, amacrine cells modulate the signals transmitted from bipolar cells to RGCs. Although S1R distribution in each subtype of the secondary neurons has not been completely delineated [45], immunostaining shows S1R presence in majority of INL cells [23, 43, 45].

Because of a paucity of experimental evidence, the function of S1R in bipolar cells is not known. Vogler et al. reported that S1R activation protects against osmotic swelling of Müller cells, but not of bipolar cells [67]. On the other hand, one-year old S1R knockout mice showed reduced amplitudes of ERG b-wave, which measures the activity of the inner retinal neurons including bipolar cells [62]. Since S1R is found in bovine photoreceptor presynaptic terminals [37], it is tempting to speculate that S1R may modulate neurotransmission to postsynaptic bipolar cells under some circumstances. There is no data available about the function of S1R in horizontal and amacrine cells. Thus, more research is needed to explore S1R functions in these secondary neurons in visual signal transmission. Such information would provide important insight into possible side effects, e.g., disturbance of synaptic transmission, of S1R-targeted interventions.

19.4.4 Photoreceptor Cells

Photoreceptors are highly specialized neurons. Through a biochemical process of phototransduction, they are capable of converting light signals into nerve impulses that eventually lead to vision (see review) [26]. There are two basic types of photoreceptors, rods and cones, each containing four morphologically and functionally distinct compartments. Rods are extremely light sensitive and responsible for night vision; cones respond to bright light and are responsible for day vision and color vision. Photoreceptors are highly susceptible to genetic defects, as well as insults from their environment. There are up to 100 photoreceptor gene loci that cause retinal diseases such as retinitis pigmentosa, a condition characterized by photoreceptor cell death (RetNet). While S1R is found in the nuclear envelope of photoreceptor cells [37], its function is not clear. Most recently, the Hara group demonstrated the importance of S1R for photoreceptors [25]. Using a 661W neuronal cell line, they found that high-affinity S1R ligand cutamesine (named SA4503) attenuated light-induced dis-

ruption of mitochondrial membrane potential and caspase-3/7 activation. Moreover, using a light-induced photoreceptor degeneration model of mice carrying a mutation in RPE65 (an RPE specific protein), cutamesine delivered by intravitreal injection partially rescued light-induced retinal dysfunction (reduced ERG) and ONL thinning. The cutamesine effect could be blocked by S1R antagonist BD1063, suggesting it was S1R specific. As photoreceptors and RPE cells are structurally and functionally dependent on each other [69], it remains unclear which cell type is the primary site of the observed S1R protective function. Of note, mechanisms of photoreceptor degeneration vary in different pathological contexts [70], and so may S1R function. Whether S1R activation is ubiquitously beneficial in the retina awaits more careful testing. Moreover, the predominance of S1R in the photoreceptor nuclear envelope raises an interesting question with regard to possible mechanisms of S1R-specific protection in photoreceptor cells. Therefore, different retinal degeneration models may be used in future experiments to comprehensively understand the role of S1R in photoreceptor pathophysiology.

19.4.5 Retinal Pigment Epithelium (RPE) Cells

RPE is a single layer of cells situated between the light-sensitive outer segments of photoreceptors and the choroid blood supply [26]. RPE possesses many functions essential to the visual process, the chief of which is to maintain photoreceptor homeostasis. Analyses of hereditary types of retinal degeneration reveal a strong dependence of RPE on photoreceptors and *vice versa*. Defects in RPE contribute to initiation and/or progression of AMD in humans (see review) [69]. Characterized by the loss of central vision, AMD is the leading cause of blindness in elderly populations, and no pharmacological treatment is available. Oxidative damage is considered as a major factor for disease onset and progression.

In situ hybridization indicated the presence of S1R mRNA in RPE [43]. However, its protein abundance and subcellular distribution in RPE cells remain unclear, partly because of the intense auto-fluorescence that masks specific S1R immunostaining. Nevertheless, in an earlier study using a human RPE cell line (ARPE-19) and adult human primary RPE cells, Bucolo et al. were able to reduce H_2O_2-induced DNA damage and cell loss by pre-treatment with PRE084, an effect abolished by S1R antagonists [71]. Most recently, using targeted siRNA screening in a human RPE1 cell line, MacVicar et al. identified S1R as a potential regulator of autophagosome homeostasis involving mitochondrial dynamics [31]. Autophagy is an important stress response pathway responsible for the removal and recycling of damaged or redundant cytosolic constituents. While autophagy is found to be an active process in the RPE in vivo [72], evidence from AMD donors indicates a decline of autophagic flux in the RPE [73]. Although not yet specifically investigated in RPE cells in vivo, S1R has been reported to influence autophagy in vitro [31, 32, 74]. A possible protective role of the S1R via autophagic regulations in RPE cells needs to be further determined experimentally. In light of an anti-oxidative function of S1R and its involvement in lipid metabolism, it appears reasonable that S1R may play a role in maintaining homeostasis of RPE cells, which are situated in a highly oxidative environment to process large amounts of lipids from phagocytosed photoreceptor membrane discs [69].

19.5 Concluding Remarks

The retina, which is composed of diverse cell types, represents a favorable model for studying the functions of S1R. In the past decade, considerable progress has been made in understanding the role of S1R in retinal degenerative diseases. While most efforts have been devoted to retinal ganglion neurons, reports on S1R in other retinal cell types are emerging. These studies generally support a protective role of S1R against stress-induced cell loss. To exploit the therapeutic potential of a S1R-targeted strategy for treating retinal diseases, more studies are required, particularly in the following areas: (1) Investigation using more diverse

pre-clinical retinal disease models for a comprehensive understanding of S1R functions. (2) Delineation of S1R-specific and non-specific effects of S1R-binding drugs, using S1R knockout animals or cells. (3) Determination of the cellular and molecular mechanisms of S1R-mediated retinal neuro-protection. (4) Evaluation of combination therapies using S1R-targeting ligands and drugs targeting other pathways. A deeper understanding of S1R-specific functions and mechanisms in the retina would lead to new therapeutic opportunities, not only for retinal diseases but also other related disorders.

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20 The Role of Sigma 1 Receptor as a Neuroprotective Target in Glaucoma

Barbara Mysona, Neil Kansara, Jing Zhao, and Kathryn Bollinger

Abstract

The role of sigma 1 receptor (S1R) in glaucoma is emerging as a promising field of study. Glaucoma is an optic neuropathy that shares common pathogenic mechanisms with other neurodegenerative diseases such as Alzheimer's and Parkinson's disease. S1R modulates multiple cellular functions associated with neurodegeneration. These include Ca^{2+} ion homeostasis, endoplasmic reticulum (ER) and oxidative stress, survival signaling pathways, neurotrophin secretion, and glial activation. S1R may also have neurorestorative properties including enhancement of neuronal plasticity and neurite outgrowth. Recent studies using agonists for S1R within the eye provide hope that it could be a therapeutic target for glaucoma. Understanding the role of S1R in glaucoma may help us to stop the progression of this sight threatening disease.

Keywords

Glaucoma • Optic nerve • Retinal ganglion cell • Neurodegeneration • Neuroprotection

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20.1 Introduction: Glaucoma a Neurodegenerative Disease

Glaucoma is the most common optic neuropathy and is the leading cause of irreversible blindness worldwide [1]. This neurodegenerative disease is characterized by retinal ganglion cell (RGC) damage. RGCs are the final output neurons that send visual information to the brain via their axons within the optic nerve. In glaucoma, there is progressive, age-associated damage and death of RGCs. Clinical signs of this degenerative process include excavation of the optic nerve head and visual field defects [2]. Elevated intraocular pressure is a major risk factor for development and progression of glaucoma [3, 4, 5, 6]. However, the mechanism by which increased IOP causes degeneration of RGCs is not known. In addition, the relationship between IOP and glaucoma pathogenesis is not absolute. A significant percentage of patients with progressive glaucoma have only mildly elevated or even normal IOP [1], yet loss of optic nerve functional and structural integrity and vision loss still occur. Conversely, many patients who have higher than average IOP do not develop glaucoma [2].

There is a fundamental need within the field of glaucoma to identify cellular targets and processes involved in RGC degeneration and regeneration. Some mechanisms have been identified, and are shared with neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [7, 8]. These common pathways include calcium ion dysregulation, oxidative damage, ER stress, mitochondrial dysfunction, and glial activation [7, 9–13]. Understanding the process of glaucomatous RGC death and the steps needed for regeneration are of increased importance in light of recent findings that some functional deficits, including visual field loss, precede permanent structural damage to the optic nerve and RGCs, and may be reversible [14–17]. Thus, factors that enhance RGC health may also be capable of reversing dysfunction, allowing RGCs to resume electrical responsiveness and transmission of visual information through the optic nerve. In addition, the important role of glial cells and their relationship in supporting RGC survival are areas of growing interest [18–22]. The ideal therapeutic target for glaucoma is one that stops the degeneration of retinal ganglion cells and re-establishes normal retinal and optic nerve head physiology, thus restoring or enhancing function of the remaining ganglion cells. Could S1R be such a target?

20.2 Sigma 1 Receptor Background: A Unique Type of Protein

S1R is a widely expressed ligand-activated receptor and molecular chaperone that was recently described as a "pluripotent modulator" [23]. Its multiple intracellular actions are consistent with its varied localization and distribution. Although S1R has been primarily localized to the ER-mitochondrion interface, or MAM (mitochondria-associated ER membrane), it is also present within the nuclear envelope and plasma membrane of some cell types [24, 25]. For example, a recent study by Mavlyutov et al., localized S1R to the nuclear envelope as well as the ER within retinal neurons including RGCs [26].

S1R is not only present in the retina. It is also expressed within the cornea, lens, iris, ciliary body, retinal pigmented epithelium (RPE), and optic nerve [27, 28]. The function of S1R within these varied ocular tissues is not fully understood. However, recent studies show that S1R acts as a molecular chaperone modulating Ca^{2+} ion release by sustaining the flux of Ca^{2+} ions from the ER to the mitochondria during periods when ER Ca^{2+} stores are depleted [25, 29, 30]. In addition, studies suggest that S1R potentiates the unfolded protein response (UPR) and enhances cellular recovery from ER stress through interaction with the ER-associated protein, Ire1 [25]. S1R is also involved in activating cellular antioxidant responses, via the Nrf2-Keap1 pathway

[31]. Furthermore, it has been shown to interact with K^{+}, Ca^{2+} and Na^{+} voltage-gated ion channels as well as NMDA receptor ion channels [32–36]. Evidence indicates that the balance of S1R's effects leads to increased cell survival and reduced cell death within several ocular tissues [37–41]. Manipulation of these cell death and survival responses is relevant to the treatment of glaucoma. In addition, S1R has been implicated in control of neurotrophin secretion, neuritogenesis, and synaptic plasticity [42–44]. These proposed roles in enhancement of neuronal function are also highly relevant to the treatment of glaucomatous neurodegeneration.

S1R can bind to a wide variety of compounds including the specific synthetic agonists (+)-pentazocine, cutamesine (SA4503) and PRE-084 as well as antagonists including NE-100 and BD1063 [45–52] In addition, S1R can bind many naturally occurring chemical substances. These include the hallucinogen N,N-dimethyltryptamine, sphingosine, cholesterol, and neurosteroids such as pregnenolone, progesterone, and didehydroepiandrosterone (DHEA) [46, 47, 49, 53, 54]. The unique ability of sigma 1 receptor to interact with diverse classes of ligands and modulate multiple cellular functions makes it a desirable therapeutic target.

The optic nerve head region is particularly susceptible to elevated IOP, which leads to axonal damage and subsequent death of RGCs [11, 14, 55]. Thus, the strong expression of S1R in the posterior regions of the eye is especially relevant to glaucoma. Posteriorly, S1R is present in the retinal pigmented epithelial cell (RPE)-choroid complex, neural retina, and optic nerve [27]. Retinal expression of S1R is particularly strong in retinal ganglion cells, glial cells, the inner nuclear layer, as well as photoreceptor cells [27, 56]. A better understanding of the role of S1R in ganglion cells and glia cells of the retina and optic nerve is essential for determining its value as a therapeutic target in glaucoma. In the following sections, we will focus on the role of S1R within the retina and optic nerve. We will also discuss examinations of S1R function within the ciliary body and trabecular meshwork, two tissues within the anterior segment of the eye that are also critical to glaucoma pathogenesis [57].

20.3 Calcium (Ca^{2+}) Homeostasis

Accumulation of calcium is a well-recognized intracellular event that can lead to neuronal injury and death [58, 59]. Regulation of intracellular calcium levels is critical to maintaining the health of retinal neurons including RGCs [60, 61] S1R is known to act as a molecular chaperone for the inositol triphosphate (IP3) receptor type 3 [29]. This membrane glycoprotein complex controls release of Ca^{2+} from intracellular stores, and ensures appropriate Ca^{2+} signaling from the ER into mitochondria [62]. Thus, sustaining the proper conformation of this receptor, via S1R-mediated chaperone activity, is an important component of Ca^{2+} homeostasis. In addition to IP3 receptors, S1R has been shown to interact with voltage gated calcium channels (VGCC) [63, 64]. In fact, within RGCs, agonists for S1R have been shown to decrease the influx of Ca^{2+} through L-type VGCC [36, 65]. This attenuation of VGCC activity may be one mechanism through which S1R functions to protect RGCs from damage.

20.4 ER and Oxidative Stress

S1R modulation of ER and oxidative stress are of great interest in glaucoma. Elevated levels of ER and oxidative stress are common characteristics of neurodegenerative diseases including glaucoma [7–10, 12]. S1R is found in the ER from which it translocates to nuclear and plasma membranes [24, 25]. In the ER, S1R forms a complex with another chaperone, BiP(Grp78), and is localized at the ER-mitochondrial interface (MAM) [25, 29]. This localization aids S1R's chaperone activities related to regulation and mediation of ER stress [25, 29, 62]. Recent studies show that sigma receptor stabilizes IRE1, an ER stress sensor involved in the unfolded protein response (UPR), with knockdown of S1R result-

ing in reduced IRE1 stress response and increased cell death [25]. S1R also mediates activation of IRE1 by mitochondrial generated reactive oxygen species (ROS) [25] suggesting that S1R may modulate responses between the ER and mitochondria.

Studies within retinal tissues and retinal neurons also support a role for S1R in modulating ER stress. In vitro studies using purified RGCs and the RGC-5 cell line, have shown that treatment with the S1R agonist, (+)-pentazocine, affects levels of ER stress proteins, including IRE1 [67] In addition, analyses of Muller glia cells derived from S1R knockout animals show changes in expression levels of several ER stress-related genes, including IRE1, Chop, and Atf6 [68].

S1R is also involved in mediation of cellular oxidative stress that occurs when production of reactive oxygen species (free radicals) exceeds the ability of antioxidant defenses to neutralize them. The essential role of S1R in modulation of oxidative stress is demonstrated in S1R knockout mice. These animals have increased levels of superoxide in liver and lung homogenates compared to wild type counterparts [69]. Additional experiments have revealed that S1R mediates cellular responses to oxidative stress by supporting activation of the antioxidant response element (ARE), which enhances production of antioxidant transcripts [69]. Within the eye, ligands for S1R have been shown to suppress production of ROS in several cell types including cultured lens cells, retinal pigment epithelial cells and retinal neurons [67, 70, 71]. In addition, Wang et al. recently reported increased ROS levels in Müller cells derived from the retinas of S1R KO animals [31]. This study also described decreased expression and activity of nuclear factor erythroid −2-related factor (NRF2) within KO-derived Muller cells. NRF2 is arguably the most important regulator of the expression of antioxidant molecules [72]. Clearly, the role of S1R in ER and oxidative stress is a critical component of its cellular function. Exploration of this aspect of S1R activity is relevant to the pathogenesis of neurodegenerative diseases including glaucoma [73–75].

20.5 Neuronal Survival Pathways

Given that retinal ganglion cell death is a predominant pathology in glaucoma that directly impacts vision loss, the prospective role of S1R in modulating neuronal survival pathways is exciting. Previous studies have shown that the S1R agonist, (+)pentazocine, protects retinal ganglion cells from death both in vitro and in vivo [38, 40, 41, 76, 77]. Hypotheses addressing the mechanisms that contribute to this protective response include S1R-mediated modulation of the anti-apoptotic protein, Bcl2. For example, Bcl2 levels are decreased under conditions of S1R knockdown in CHO cells [78]. In addition, work reported by Ha et al., described decreased levels of Bcl2 within the retinas of S1R KO mice [68]. Recent investigations have also evaluated genes that regulate Bcl2 expression. ERK1/2 signaling is well known to control Bcl2 levels, and ERK1/2 activation was also reduced in retinas of S1R KO mice [68]. In addition, a recent study reported by Mueller et al., found that treatment of purified RGCs with the S1R agonist, (+)-pentazocine, increases ERK1/2 phosphorylation [40]. The (+)-pentazocine-induced, increased ERK1/2 phosphorylation protected cultured RGCs from ischemia-mediated cell death [40]. These results are consistent with recent in vivo studies which found that (+)-pentazocine treatment enhanced retinal phospho-ERK1/2 levels and protected RGCs in mice subjected to excitotoxicity via intravitreal NMDA injection [41]. Overall, studies suggest that S1R activities affect neuronal survival pathways and that these actions may be relevant to glaucomatous neurodegeneration.

20.6 Growth Factor Secretion and Neurite Outgrowth

BDNF (brain derived neurotrophic factor) is a powerful neurotrophin and one of the most important factors in neuronal differentiation and survival [79, 80]. Scarcity of this neurotrophin, which is critical for the survival of RGCs, has been implicated in glaucoma pathogenesis [81, 82]. S1R agonists are have been shown to increase

release of brain derived neurotrophic factor (BDNF) from brain (hippocampus) tissue and from neuronal and astrocyte cell cultures [42, 83, 84]. Studies done in vitro suggest that increased BDNF release results from S1R-dependent enhancement of the post-translational processing of BDNF [42]. In addition, Kimura et al. reported that S1R interacts with with the BDNF receptor, TrkB, and promotes neurite elongation in cerebellar granule neurons [43]. These authors also demonstrated that treatment with S1R agonist results in increased binding of S1R to TrkB, as demonstrated by coimmunoprecipitation experiments. Therefore, S1R likely plays an important role in neuronal differentiation and plasticity. Since BDNF and associated pathways are critical to neuronal survival, the neuroprotective effects of S1R agonists may be in part due to S1R-mediated upregulation of BDNF.

20.7 Glial Effects

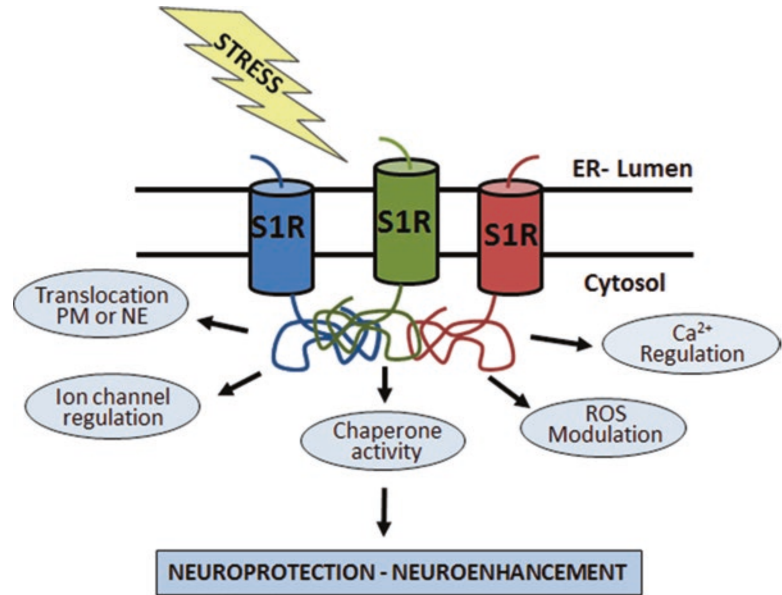
In addition to direct neuroprotective effects, ligands for S1R have been shown to influence glial responses to neurotoxic stimuli [85, 86]. Within the retina, S1Rs have been localized to Müller cells, microglia, and astrocytes [27, 85]. S1Rs modulate the activation of both CNS-derived and retina-derived primary microglia [85, 86]. In addition, ligands for S1R affect retinal Müller glia cells by influencing release of inflammatory cytokines and by regulating their ER and oxidative stress responses [31, 69, 87]. Furthermore, agonist-mediated activation of S1R has been shown to increase release of BDNF from brain-derived primary astrocytes [84]. Given the known effects of S1R activation on glia cells, it is possible that agonists for S1R protect RGCs from injury via mechanisms that involve modulation of glia responses. S1R-mediated changes to glial activities may indirectly protect RGCs through changes in cytokine or neurotrophic factor release.

20.8 Intraocular Pressure

Most examinations of S1R within the eye have focused on its role in the retina and optic nerve. Within these ocular tissues, ligands for S1R may offer a strategy for directly protecting RGCs from glaucomatous damage. The mechanism of action for a direct effect might involve S1R-mediated regulation of ER or oxidative stress within RGCs themselves (as discussed above). These ligand-mediated effects could protect RGCs from damage independent of IOP. This would provide a novel and important treatment strategy for glaucoma.

S1R is also expressed in the ciliary body and the region occupied by the trabecular meshwork [27, 28]. These two ocular tissues are involved in the regulation of IOP. Relative to retina and optic nerve, less is known about the role of S1R within these tissues. However, some studies indicate that activation of S1R reduces IOP, though the mechanism and exact conditions under which this occurs are uncertain [88, 89]. For example, work by Bucolo and colleagues showed that topical administration of (+)-pentazocine reached intraocular tissues within 30 min [28, 88]. This treatment resulted in an immediate S1R-specific reduction of IOP in both control and hypertensive eyes that returned to pretreatment values after 4 h [28]. These results suggest a role for S1R in short-term modulation of intraocular pressure. However, which cell type or combination of cellular mechanisms is effecting the observed reduction in IOP is unknown. In addition, Ha et al. measured IOP in S1R KO mice and found no significant difference between S1R KO animals and their WT counterparts [90]. These results suggest that S1R does not play a direct role in modulation of IOP. Overall, some studies suggest that in addition to direct RGC neuroprotective effects, S1R may affect glaucomatous neurodegeneration indirectly, through an effect on IOP level. Definitely much remains to be discovered about the role of sigma 1 receptor in regulating intraocular pressure.

Fig. 20.1 Sigma receptor (SR-1) response to stress leads to neuroprotection and neuroenhancement



20.9 Sigma 1 Receptor as a Therapeutic Target: Conclusions and Future Directions

S1R is a powerful neuroprotective and neuroenhancing protein that can be activated using ligands that are already undergoing human clinical trials [91]. S1R agonists preserve neuronal structure and function in animal models of Amyotrophic Lateral Sclerosis (ALS), Alzheimers Disease, Parkinson's Disease, and Huntington's disease [44, 92–94]. In addition, treatment with S1R ligands has been shown to promote brain plasticity and functional recovery in animal models of stroke, and to ameliorate cognitive impairment through enhanced synaptic transmission and neurogenesis within the hippocampus [95, 96]. Furthermore, several *in vitro* studies have described the pleiotropic neuroprotective activities of this multitasking protein (Fig. 20.1) [38, 40, 65, 97]. These studies suggest that mechanisms for neuroprotection mediated by S1R include regulation of Ca^{2+} homeostasis, modulation of ER and oxidative stress, activation of neuronal survival pathways, enhancement of neurotrophin release, regulation of glial activity, and effects on intraocular pressure. Since most analyses investigating the mechanism of S1R function utilize cell lines and overexpression systems, a great need exists for more in vivo studies to examine the action of S1R in primary cells, animal models, and in specific diseases such as glaucoma. Future studies should be directed toward in vivo evaluation of S1R under glaucomatous conditions including ocular hypertension.

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