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# The ALS-Related $\sigma$ 1R E102Q Mutant Eludes Ligand Control and Exhibits Anomalous Response to Calcium

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**Abstract:** Sigma receptor type 1 ( $\sigma$ 1R) is a transmembrane protein expressed throughout the central nervous system and in certain peripheral tissues. The human  $\sigma$ 1R E102Q mutation causes juvenile amyotrophic lateral sclerosis (ALS), likely by inducing a series of alterations in calcium efflux from the endoplasmic reticulum (ER) to mitochondria that affects calcium homeostasis and cellular survival. Here, we report the influence of calcium on  $\sigma$ 1R E102Q associations with glutamate *N*-methyl-D-aspartate receptors (NMDARs), binding immunoglobulin protein (BiP), and transient receptor potential calcium channels A1, V1, and M8. The mutant protein inhibited the binding of calmodulin to these calcium channels and interacted less with BiP than wild-type  $\sigma$ 1R, thereby contributing to calcium homeostasis dysfunction. Mutant  $\sigma$ 1R, but not wild-type  $\sigma$ 1R, strongly bound to histidine triad nucleotide binding protein 1, which regulates neuromuscular synaptic organization and target selection through teneurin 1. While ligands regulated the association of  $\sigma$ 1R wild-type with NMDARs and BiP, they failed to modulate the interaction between these proteins and the  $\sigma$ 1R E102Q mutant. Thus, the  $\sigma$ 1R E102Q mutant exhibited an anomalous response to cytosolic calcium levels, altered affinity for target proteins, and a loss of response to regulatory ligands. We believe that these modifications may contribute to the onset of juvenile ALS.

**Keywords:** sigma type receptor 1; juvenile amyotrophic lateral sclerosis; E102Q mutation; *N*-methyl-D-aspartate receptor; transient receptor potential calcium channels; binding immunoglobulin protein

## 1. Introduction

The sigma receptor type 1 ( $\sigma$ 1R) is a 223-amino-acid polypeptide that is widely distributed in both the central and peripheral nervous systems [1–4]. This protein is encoded by the *SIGMAR1* gene and was initially described as an opioid receptor on the plasma membrane of neurons [5–7]. Later,  $\sigma$ 1R was also found in the endoplasmic reticulum (ER) [8] and the nuclear envelope [9,10].  $\sigma$ 1R participates in several processes, such as neuronal survival, ion channel activity,  $\text{Ca}^{2+}$  signaling, synaptic plasticity, memory, and drug addiction [11–13]. Furthermore, it has also been implicated in central nervous system pathologies, including amnesia [13], pain [14], depression [15], schizophrenia [16], stroke [17], retinal neuron degeneration [18,19], and Alzheimer's [20], Parkinson's [21], and Huntington's [22] diseases.

Different disorders are associated with known mutations in human *SIGMAR1*, including frontotemporal lobar degeneration (FTLD) [23] and motor neuron diseases such as autosomal recessive distal hereditary motor neuropathy [24] and juvenile amyotrophic lateral sclerosis (ALS) [25,26].  $\sigma$ 1R is enriched in motor neurons in the brainstem and spinal cord [27]. Accordingly,  $\sigma$ 1R knockout mice exhibit deficits in motor

control caused by motor neuron degeneration in the spinal cord [28], and loss of  $\sigma$ 1R exacerbates ALS progression in G93A-SOD1 mice [26].  $\sigma$ 1R establishes  $\text{Ca}^{2+}$ -dependent associations with a series of signaling proteins, such as binding immunoglobulin protein (BiP), at mitochondria-associated ER membranes (MAMs). Upon ER  $\text{Ca}^{2+}$  depletion or ligand stimulation,  $\sigma$ 1R dissociates from BiP, leading to prolonged  $\text{Ca}^{2+}$  influx into mitochondria via inositol 1,4,5-triphosphate receptor type 3 (IP3R3) [11]. A single missense mutation in the second exon of the *SIGMAR1* gene is associated with the juvenile form of ALS [25]. This mutation, which involves the substitution of glutamine for glutamic acid at position 102 (E102Q) of  $\sigma$ 1R, is located in the linker region between  $\beta$ 2 and  $\beta$ 3 [29]. A series of recent studies have shed some light on the molecular mechanisms by which the  $\sigma$ 1R E102Q mutation causes ALS. In vitro, the  $\sigma$ 1R E102Q mutant is prone to aggregate, and in transfected cells, the mutant is unstable and incapable of binding to IP3R3 in the ER membrane [26]. Moreover,  $\sigma$ 1R E102Q accumulates in the ER and associated compartments, provoking alterations in proteasomal degradation and calcium homeostasis [30].

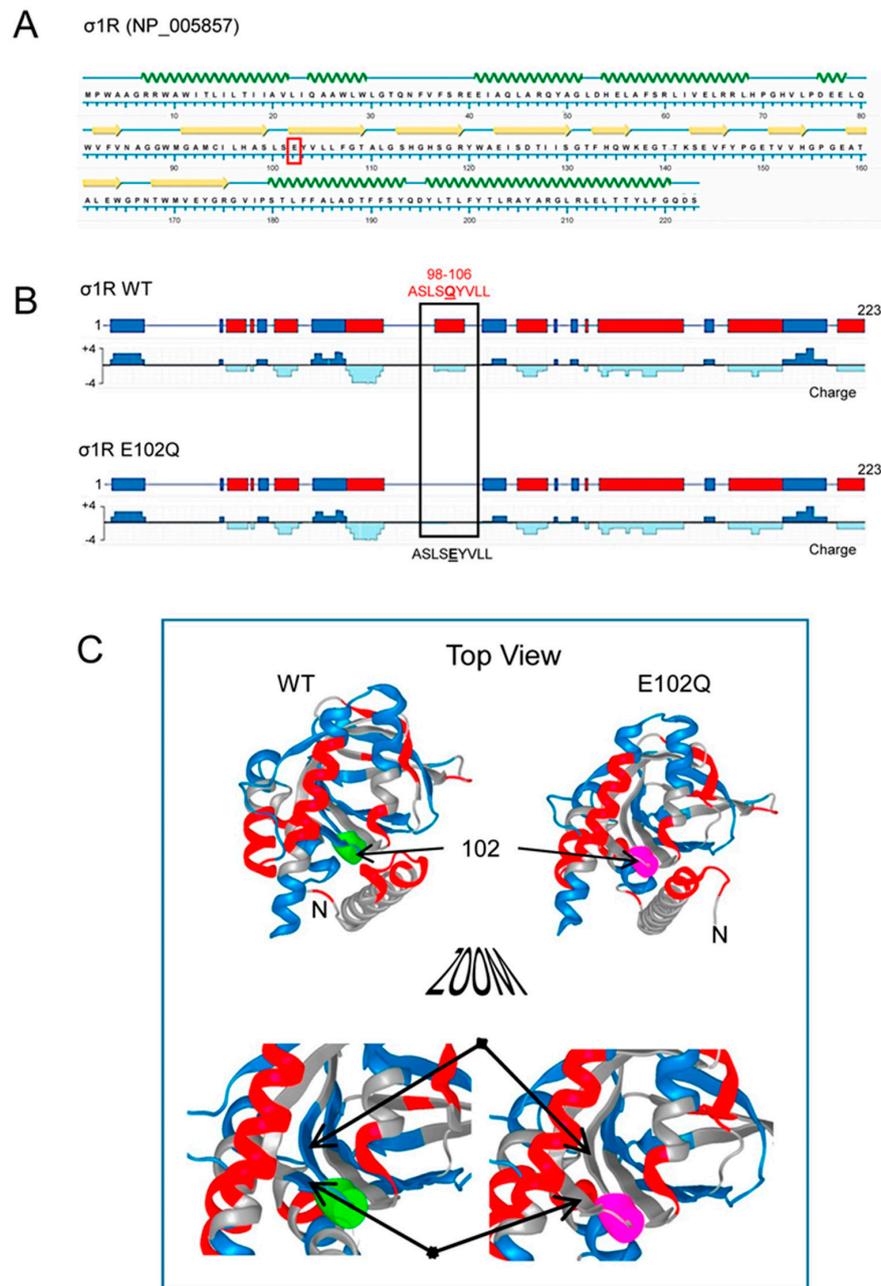
Different classes of calcium channels in the plasma membrane dynamically control intracellular calcium levels, such as tetrameric glutamate *N*-methyl-D-aspartate receptors (NMDARs) and transient receptor potential (TRP) family channels. Notably,  $\sigma$ 1R binds to the NR1 subunit of ionotropic glutamate NMDARs [31,32] and, together with histidine triad nucleotide-binding protein 1 (HINT1), coordinates the activity of G-protein coupled receptors (GPCRs), such as the mu-opioid receptor (MOR), with that of NMDARs [32]. A similar mechanism also applies to neural TRP ankyrin member 1 (TRPA1), TRP vanilloid member 1 (TRPV1), and TRP melastatin member 8 (TRPM8). Recent data showed that  $\sigma$ 1R and Calmodulin (CaM) bound directly to cytosolic regions of these TRP channels [33]. The binding of  $\sigma$ 1R to proteins such as the C1 cytosolic region of NMDAR's NR1 subunit (NR1-C1) [31,32], TRP channels [33], and the protein BiP in the ER [11] is regulated by calcium. An increase in the calcium level promotes the interaction between  $\sigma$ 1R and these third partner signaling proteins, while calcium depletion hinders these associations.

Specifically,  $\sigma$ 1R competes with  $\text{Ca}^{2+}$ -activated CaM and HINT1 to bind to the cytosolic C0-C1-C2 region of the NR1 subunit [31] and TRP channels [33]. As a result,  $\sigma$ 1R promotes the activity of these calcium channels, while HINT1 and particularly  $\text{Ca}^{2+}$ -CaM diminish their activity. While there is no cure for ALS, its progression can be delayed by drugs such as riluzole, which hinders the function of NMDARs [34]. It is possible that the  $\sigma$ 1R E102Q mutant exacerbates NMDAR activity in motor neurons, where  $\sigma$ 1R is particularly abundant [27].

These observations prompted us to study whether human  $\sigma$ 1R E102Q mutant establishes dysregulated interactions with plasma membrane proteins that influence cytosolic  $\text{Ca}^{2+}$  levels. Thus, BiP, the NR1-C1 subunit of NMDARs, the MOR, the HINT1 protein, and the N- and C-terminal cytosolic regions of TRPA1, TRPV1, and TRPM8 channels were selected for in vitro assays. Whether the interactions between the  $\sigma$ 1R E102Q mutant and various signaling proteins are regulated by calcium levels and the capacity of  $\sigma$ 1R ligands to modify the interaction between the  $\sigma$ 1R mutant and the NR1 subunit or BiP were also investigated. Compared to wild-type (WT)  $\sigma$ 1R, the E102Q mutant receptor exhibited a stronger association with its partner proteins, except for BiP and TRPV1 C terminus (Ct), which interacted with the E102Q mutant at a much lower level than with WT  $\sigma$ 1R. Moreover, the interactions between  $\sigma$ 1R E102Q and its partner proteins were much less dependent on  $\text{Ca}^{2+}$  than those between WT  $\sigma$ 1R and the same proteins, and the  $\sigma$ 1R ligands studied did not disrupt the association of  $\sigma$ 1R E102Q with NR1 subunits or BiP protein.

## 2. Results

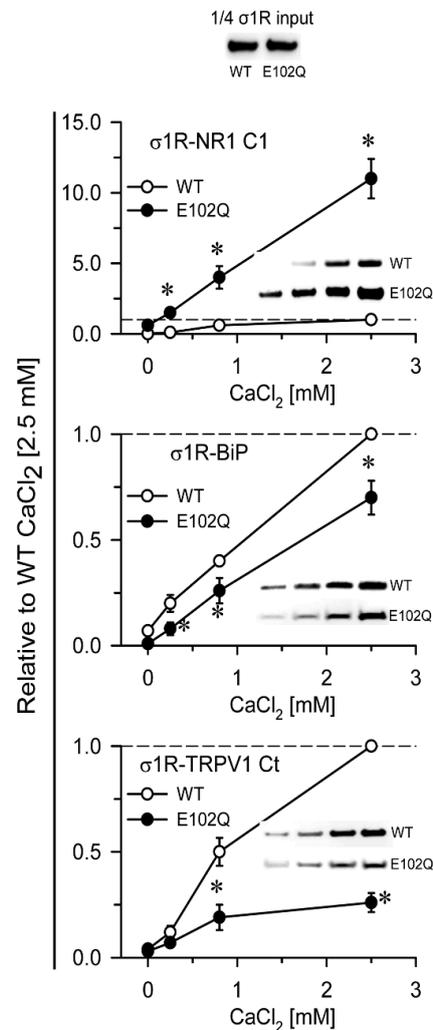
Human  $\sigma$ 1R is composed of 223 amino acids that form seven helices and ten  $\beta$ -sheets, with the rest of the protein being linear sequences (Figure 1A; NovaFold v17, DNASTAR). The  $\sigma$ 1R E102Q mutant lacks a negatively charged cluster located in the linker region between  $\beta$ 2 and  $\beta$ 3 (residues 98–106, ASLSEYVLL), which may be essential for electrostatic interactions with partner proteins (Figure 1B,C).



**Figure 1.** Secondary structure, charge distribution map, and 3D model of sigma receptor type 1 ( $\sigma$ 1R). (A) Protein sequence and secondary structure of  $\sigma$ 1R, with helices indicated by green waves and  $\beta$ -sheets by yellow arrows. The amino acid change in human  $\sigma$ 1R associated with the juvenile form of amyotrophic lateral sclerosis (ALS) is indicated in red. (B) Charge distribution map of wild-type (WT)  $\sigma$ 1R and  $\sigma$ 1R E102Q (the images were created using NovaFold v17, DNASTAR, Inc., Madison, WI, USA). Positive charges are indicated by blue and negative charges are indicated by red. The E102Q mutant protein lacks a negatively charged cluster located in the linker region between  $\beta$ 2 and  $\beta$ 3 (residues 98–106, ASLSQYVLL). (C) 3D structure of  $\sigma$ 1R showing the WT and mutated amino acid as a colored tube. E102 is indicated by green and Q by pink. The structural model of  $\sigma$ 1R and its secondary structure shown here were generated with NovaFold v. 17 (DNASTAR).

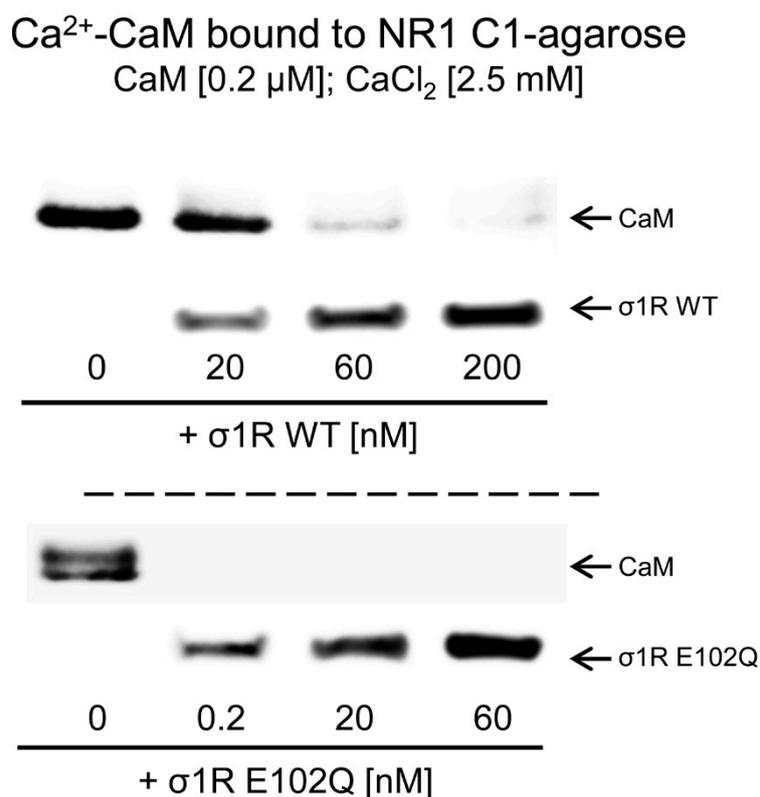
In *in vitro* assays,  $\sigma$ 1R interacts with a series of signaling proteins, such as the cytosolic C0-C1-C2 region of the NR1 subunit of glutamate [31,32], BiP [11], the C-terminal sequence of MOR [32], and the N- and C-terminal cytosolic domains of TRPA1, TRPM8, and TRPV1 channels [33] in a

calcium-dependent manner. Increases in calcium levels promote the interaction of  $\sigma$ 1R with third partner signaling proteins, while calcium depletion inhibits these associations. The human E102Q mutant also exhibited calcium-dependent associations with the NR1-C1 subunit, BiP, and the C-terminal sequence of TRPV1.  $\sigma$ 1R E102Q and WT  $\sigma$ 1R differed in their binding affinity for these proteins. The mutant had a much higher affinity than the WT for the NR1-C1 subunit, while the opposite was observed for BiP and TRPV1 Ct (Figure 2).



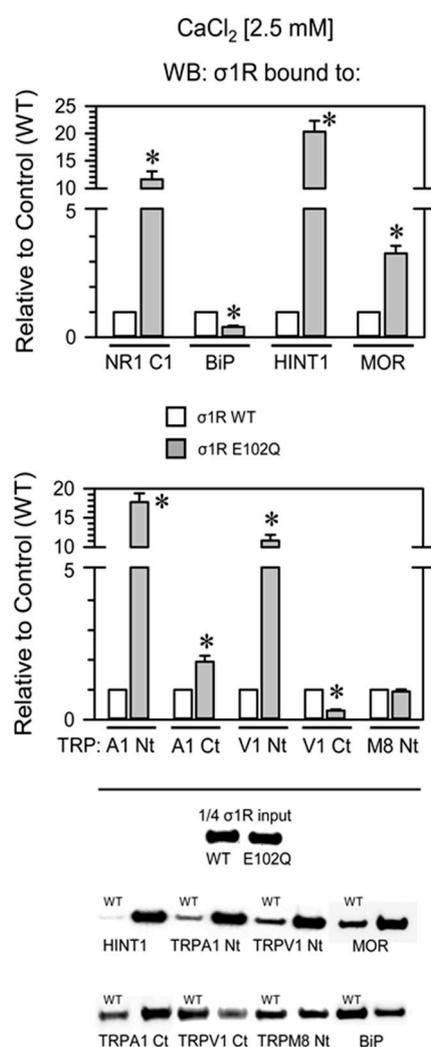
**Figure 2.** Calcium-dependent binding of  $\sigma$ 1R WT and  $\sigma$ 1R E102Q to the NMDAR NR1 subunit, BiP, and TRPV1 Ct. Recombinant C0-C1-C2 of the NR1 subunit, BiP, and TRPV1 Ct were covalently attached to N-Hydroxysuccinimidy (NHS)-activated Sepharose® and incubated with 100 nM WT  $\sigma$ 1R or its mutant form in the presence of increasing amounts of calcium chloride (0, 0.25, 0.83, and 2.5 mM). The prey proteins alone (negative controls) did not bind to NHS-activated Sepharose. The pellets obtained were processed to measure  $\sigma$ 1R expression by western blotting (see Section 4 and Figure S1). The assays were performed at least twice, and each sample was analyzed in duplicate. The association of WT  $\sigma$ 1R or  $\sigma$ 1R E102Q with the covalently attached proteins (NR1, BiP, and TRPV1) at different concentrations of calcium is shown relative to the association of WT  $\sigma$ 1R with the proteins in the presence of 2.5 mM calcium (assigned an arbitrary value of 1). \* indicates significant differences compared to the WT  $\sigma$ 1R group and the corresponding concentration of calcium; all data were analyzed by ANOVA followed by the pairwise Holm–Sidak multiple comparison test;  $p < 0.05$ ;  $\sigma$ 1R, sigma receptor type 1; NMDAR, *N*-methyl-D-aspartate receptor; NR1-C1, cytosolic region of the C0-C1-C2 domains of the NR1 subunit of NMDARs; BiP, binding immunoglobulin protein; TRPV1 Ct, cytosolic C-terminal domain of transient receptor potential calcium channel type V1; WT, wild-type.

The activation of CaM by calcium rapidly regulates different signaling pathways and the activities of various proteins, such as NMDARs and TRP channels. The CaM-binding motifs in the NMDAR NR1-C1 subunit and TRPA1, TRPV1, and TRPM8 overlap with the binding sites of  $\sigma$ 1R, and thus,  $\sigma$ 1R competes with CaM for binding to specific sequences in the cytosolic regions of these proteins [31,33]. We observed that, in the presence of 2.5 mM  $\text{CaCl}_2$ , increasing concentrations of WT  $\sigma$ 1R and the E102Q mutant reduced the binding of calcium-activated CaM to the cytosolic C0-C1-C2 region of the NR1 subunit. While binding of 200 nM CaM was abolished by 60 nM WT  $\sigma$ 1R, the E102Q mutant, at a concentration less than 0.2 nM, prevented CaM binding to the NR1 protein (Figure 3).



**Figure 3.** Competition analysis of  $\sigma$ 1R and CaM for binding to the NR1-C1 subunit of NMDARs. CaM (0.2  $\mu$ M) was incubated with the NR1-C1 subunit, which was covalently attached to NHS-activated Sepharose® in the presence of 2.5 mM  $\text{CaCl}_2$  and increasing concentrations of WT  $\sigma$ 1R or the human  $\sigma$ 1R E102Q mutant. After incubation, CaM and  $\sigma$ 1R that remained bound to the NR1-C1 subunit were isolated, resolved by SDS-PAGE and analyzed by western blotting. The assays were repeated at least twice, and comparable results were obtained. Representative blots are shown. CaM, calmodulin;  $\sigma$ 1R, sigma receptor type 1; WT, wild-type.

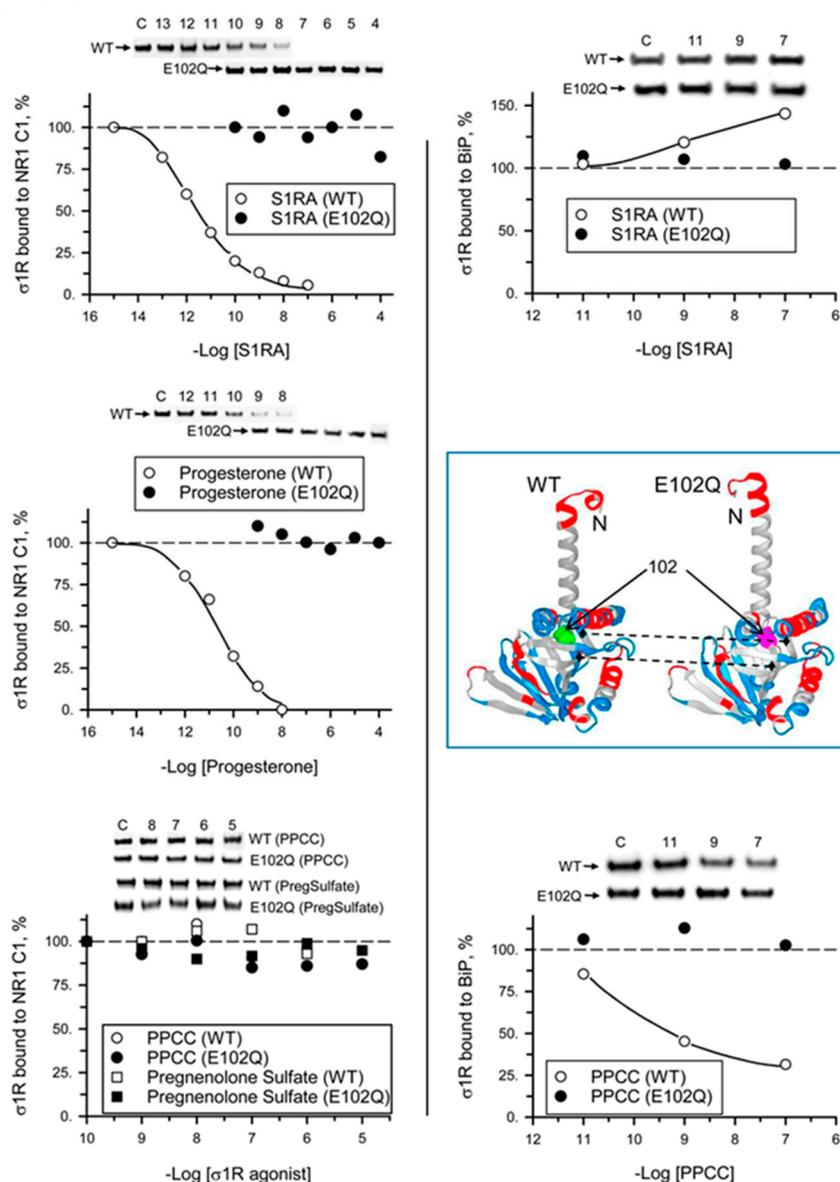
Compared with WT  $\sigma$ 1R, the E102Q mutant bound more strongly to the NR1-C1 subunit, the HINT1 protein, and the N-terminal domains of TRPA1 and TRPV1 and with lower affinity to the C-terminal sequences of MOR and TRPA1. The opposite was observed for BiP and the C-terminus of TRPV1, and both forms of  $\sigma$ 1R displayed similar binding to the N-terminus of TRPM8 (Figure 4).



**Figure 4.** Binding of WT  $\sigma$ 1R and  $\sigma$ 1R E102Q to proteins that regulate NMDAR function and to the cytosolic domains of TRPA1, TRPV1, and TRPM8. Recombinant cytosolic C0-C1-C2 region of the NMDAR NR1 subunit, the C-terminal cytosolic region of MOR, the HINT1 protein, BiP, and the N- and C-terminal cytosolic domains of TRPA1, TRPV1, and TRPM8 channels were covalently attached to agarose and incubated with human WT  $\sigma$ 1R or its mutant in the presence of 2.5 mM CaCl<sub>2</sub> (details as in Figure 2). The assays were repeated at least twice, and comparable results were obtained. Representative blots are shown. For each interaction between  $\sigma$ 1R WT or the  $\sigma$ 1R E102Q mutant and a given protein, \* indicates a significant difference compared to the  $\sigma$ 1R WT group (assigned an arbitrary value of 1); all data were analyzed by ANOVA followed by the pairwise Holm–Sidak multiple comparison test;  $p < 0.05$ ;  $\sigma$ 1R, sigma receptor type 1; NR1-C1, cytosolic region the C0-C1-C2 domains of the NR1 subunit of NMDARs; BiP, binding immunoglobulin protein; HINT1, histidine triad nucleotide binding protein 1; MOR, mu-opioid receptor; TRP, cytosolic transient receptor potential types A1, V1, and M8; Ct, C-terminal domain; Nt, cytosolic N-terminal domain; WT, wild-type.

We analyzed the ability of  $\sigma$ 1R ligands to regulate the interactions of mutant  $\sigma$ 1R with the cytosolic C0-C1-C2 region of the NMDAR NR1 subunit and with BiP in the presence of 2.5 mM CaCl<sub>2</sub>. While  $\sigma$ 1R antagonists such as progesterone and S1RA greatly reduced the  $\sigma$ 1R WT-NR1 subunit interaction in a concentration-dependent manner [35], they did not have an effect on the  $\sigma$ 1R E102Q-NR1 subunit interaction. The agonists PPCC and pregnenolone sulfate did not significantly alter the interaction between WT  $\sigma$ 1R or E102Q and the NR1 subunit. On the other hand, S1RA augmented

and PPCC reduced the association of WT  $\sigma$ 1R with BiP; however, these ligands failed to alter the  $\sigma$ 1R E102Q–BiP interaction (Figure 5).



**Figure 5.** Effect of  $\sigma$ 1R ligands on the interactions of WT  $\sigma$ 1R and the E102Q mutant with the NR1-C1 subunit and BiP. Recombinant C0-C1-C2 region of NR1 and BiP were covalently attached to NHS-activated Sepharose® and incubated with WT  $\sigma$ 1R or mutant  $\sigma$ 1R (100 nM). Unbound  $\sigma$ 1R was removed by three cycles of washing/resuspension. The protein– $\sigma$ 1R complexes were incubated for 30 min with rotation at room temperature (RT) in the presence of increasing concentrations of  $\sigma$ 1R ligands in a final volume of 300  $\mu$ L (50 mM Tris-HCl, pH 7.5, and 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)), and 2.5 mM CaCl<sub>2</sub> was used throughout the procedure. Finally,  $\sigma$ 1R that remained attached to the NR1 subunit or BiP was resolved by SDS-PAGE and evaluated by immunoblotting. The assays were performed two times, and the samples were analyzed in duplicate for each ligand concentration. Representative blots are shown. Inset, WT  $\sigma$ 1R and the E102Q mutant showing position 102 (solid arrows) and the loss of a negative charge in the mutant surrounding the Q residue (dashed arrows) (Novafold v. 17; DNASTAR Inc., Madison, WI, USA).

### 3. Discussion

$\sigma$ 1R physically interacts with a series of cytosolic signaling proteins associated with the ER and mitochondrial calcium regulation, such as ankyrin B, IP3R3, and BiP [11,36]. At the mitochondria-associated ER membrane,  $\sigma$ 1R associates with BiP, and depletion of ER calcium or ligand binding to  $\sigma$ 1R disrupts this association, leading to prolonged calcium-mediated cellular signaling in mitochondria via IP3R3 [11]. Thus,  $\sigma$ 1R deficiency results in deregulation of calcium homeostasis at the ER–mitochondria interface through mislocalization of IP3R3. Notably, in N2a-IP3R3 cells,  $\sigma$ 1R E102Q does not interact with IP3R3, and upon ATP stimulation, the regulatory effect of the ALS-linked mutant on calcium release into the cytoplasm and mitochondria upon ATP stimulation is completely abolished [26].

In our study, the  $\sigma$ 1R E102Q mutant showed an anomalous dependence of calcium levels to establish associations with target proteins. Thus, at low calcium levels, WT  $\sigma$ 1R showed little or no association with the NR1 subunit, but the E102Q mutant strongly bound to this protein. Differences in competition with calcium-activated CaM for binding to the NR1 subunit were also observed between WT  $\sigma$ 1R and the mutant. These observations indicated that  $\sigma$ 1R E102Q prevailed over the NMDAR inhibitor  $\text{Ca}^{2+}$ -CaM in binding to the NR1 subunit [37], thus increasing the entrance of calcium toward the cytosol.  $\sigma$ 1R in the ER membrane binds BiP in a calcium-dependent manner, and this binding is retained through the  $\text{Ca}^{2+}$ -regulated C-terminal domain (residues 112–223) of  $\sigma$ 1R [11]. Our study indicated that  $\sigma$ 1R E102Q had lower affinity for BiP than WT  $\sigma$ 1R, which is consistent with the *in vivo* finding that  $\sigma$ 1R E102Q poorly colocalizes with the BiP protein [38]. Thus,  $\sigma$ 1R E102Q mutation may also increase calcium flux into mitochondria, contributing to the deregulation of intracellular calcium homeostasis.

It has been described that the binding of the  $\sigma$ 1R to TRPs may favor the open probability of the channel, while CaM will reduce TRP activity by competing and diminishing  $\sigma$ 1R binding. Hence, the resulting activity of the TRP calcium channels may depend on the concentrations of CaM and  $\sigma$ 1R in their cytosolic environment [33]. We found that the influence of calcium on the formation of the  $\sigma$ 1R E102Q–TRPV1 Ct complex was different than that on the formation of the WT  $\sigma$ 1R–TRPV1 Ct complex; however, the influence that such a modification has on intracellular calcium signaling requires further research. Similarly, the  $\sigma$ 1R mutant may block the ability of  $\text{Ca}^{2+}$ -CaM to access the regulatory N-terminal cytosolic sequences of TRPA1 and TRPV1, through which CaM mainly reduces the influx of extracellular calcium [33]. However,  $\sigma$ 1R promotes the binding of CaM to TRPA1 Ct [33], and  $\sigma$ 1R E102Q may moderately enhance the binding of  $\text{Ca}^{2+}$ -CaM to this cytosolic region. On the other hand, the mutant increased its association with TRPV1 N terminus (Nt), but reduced its binding to the C-terminal sequence of TRPV1. Overall,  $\sigma$ 1R E102Q deregulated the binding of  $\text{Ca}^{2+}$ -CaM to TRPA1 and TRPV1 channels.

On the other hand, the affinity of the  $\sigma$ 1R E102Q mutant for MOR and especially the HINT1 protein was higher than that of WT  $\sigma$ 1R. A series of GPCRs, such as the MOR, regulate NMDARs, inducing glutamate neural activity [35]. Thus, the HINT1– $\sigma$ 1R protein complex links the activity of MOR with that of NMDARs [31,32].  $\sigma$ 1R impairs the ability of  $\text{Ca}^{2+}$ -CaM to reduce the opening probability of NMDARs and consequently to inhibit calcium influx [37]. Therefore,  $\sigma$ 1R promotes NMDAR activity, while  $\text{Ca}^{2+}$ -CaM and HINT1 diminish NMDAR activity. The human  $\sigma$ 1R E102Q mutant may deregulate the interactions of NMDARs with other signaling proteins, contributing to neurological disorders, including neurodegenerative diseases [39], and likely to alterations in motor coordination. The progression of ALS can be delayed by drugs that inhibit the function of NMDARs [34]. Interestingly, there was a strong association between HINT1 and the  $\sigma$ 1R E102Q mutant. The WT  $\sigma$ 1R barely forms stable associations with HINT1 [31,32], and thus, the mutant may alter the physiologically stable associations of HINT1 with other signaling proteins. HINT1 is a zinc- and CaM-regulated SUMO protease [40], and a series of HINT1 mutants have been reported to cause human autosomal recessive axonal neuropathy with neuromyotonia [41]. The association of the  $\sigma$ 1R mutant with the HINT1 protein is relevant to the normal functioning of motor pathways. HINT1 couples with the intracellular domain of teneurin 1, which acts as a transcription factor in the

nucleus [42]. The teneurin family (four members in humans, Ten1–4) promotes neurite outgrowth, cell adhesion, dendritic morphology, axonal guidance, and synapse formation and regulates neuromuscular synapse organization and target selection [39,43]. Mutations in the human *TEN4* gene are associated with essential tremor movement disorder in patients [44]. *Ten4*<sup>-/-</sup> mice exhibit a tremor-like phenotype, and a missense mutation in the *TEN1* gene is related to disorders affecting movement and posture [45].

$\sigma$ 1R is highly expressed in motor neurons [1,27], and autosomal recessive loss-of-function mutations in  $\sigma$ 1R are primarily associated with distal hereditary motor neuropathy [10,46,47], and ALS/FTLD [23,48]. In vitro studies have revealed aberrant subcellular distribution of  $\sigma$ 1R E102Q in NSC34 cells and have shown that cells expressing the mutant protein are more prone to apoptosis induced by ER stress than those expressing the WT protein [25]. Moreover, expression of the  $\sigma$ 1R E102Q mutant protein reduces mitochondrial ATP production, inhibits proteasome activity, and causes mitochondrial injury, aggravating ER stress-induced death of neuro2A cells [49]. Additionally, in cultured hippocampal neurons, overexpression of the  $\sigma$ 1R E102Q mutant destabilizes mushroom spines [50]. The  $\sigma$ 1R E102Q mutant protein aggregates and accumulates in the ER and associated compartments in transfected cells, provoking alterations in proteasomal degradation and calcium homeostasis [30]. Given the functional relation between HINT1 and  $\sigma$ 1R, alterations in the normal functioning of any of these proteins may contribute to the onset of motor neuron pathology.

Various exogenous ligands and neurosteroids alter the calcium-dependent association of  $\sigma$ 1R with regulated proteins [11,31,33,36]. Depending on the interacting protein, the same  $\sigma$ 1R ligand can either promote the disruption of the complex or prevent the disrupting activities of other ligands [33]. Thus,  $\sigma$ 1R ligands exhibit biased activity to regulate subsets of  $\sigma$ 1R interactions with third partner proteins, which can be exploited for the development of site-specific drugs with therapeutic significance.  $\sigma$ 1R lacks a GPCR structure and transduction regulation; thus, it is considered as a ligand-operated chaperone [51]. While S1RA and progesterone diminished the association of  $\sigma$ 1R with the NR1 subunit, these antagonists failed to alter the association of this subunit with  $\sigma$ 1R E102Q. S1RA enhanced and PPCC diminished the  $\sigma$ 1R–BiP interaction; however, these ligands did not alter the  $\sigma$ 1R E102Q–BiP interaction.

E102 forms a hydrogen bond with Y173 in  $\beta$ 10 [52] and a pair of hydrogen bonds with the backbone amide nitrogen atoms of V36 and F37, which are part of a structured tether between the transmembrane domain and cytosolic domain. The E102Q mutation abolishes the negatively charged cluster located in the linker region between  $\beta$ 2 and  $\beta$ 3 (residues 98–106, ASLSEYVLL) and alters hydrogen-bonding properties at the junction between the N-terminal helix and the C-terminal domain adjacent to the ligand-binding pocket of  $\sigma$ 1R [29]. This mutation does not impede the binding of ligands to  $\sigma$ 1R, as observed in ligand binding assays [52], but modifies the affinity of  $\sigma$ 1R for target proteins and its response to cytosolic calcium levels. It also abolishes the effects of ligand binding on the formation of complexes between  $\sigma$ 1R and regulated signaling proteins.  $\sigma$ 1R E102Q binds poorly to BiP in the ER and may promote the influx of extracellular calcium through calcium channels such as NMDARs and certain types of TRP channels, contributing to the disruption of cellular calcium homeostasis and the onset of juvenile ALS.

## 4. Materials and Methods

### 4.1. Recombinant Protein Expression

The coding region of full-length murine  $\sigma$ 1R (AF004927) and its mutated sequence, HINT1 (NM\_008248), the C-terminal region of MOR1 (AB047546: residues 286–398), the C0–C1–C2 region of the glutamate NMDAR NR1 subunit (NM\_008169: residues 834–938), and the N- and C-terminal regions of TRPA1 (NP\_808449; residues 1–721 and 961–1125, respectively), TRPV1 (NP\_542437; residues 1–433 and 680–839, respectively), and TRPM8 (NP\_599013; residues 1–639) were amplified by RT-PCR using total RNA isolated from the mouse brain as a template.

Specific primers containing an upstream Sgf I restriction site and a downstream Pme I restriction site were used as described previously [31]. The PCR products were cloned downstream of the glutathione S-transferase (GST)/HaloTag coding sequence (Flexi Vector, Promega Biotech Iberica, Madrid, Spain) and the tobacco etch virus (TEV) protease site, and sequencing revealed that the sequences of the proteins were identical to the GenBank sequences. The vectors were introduced into *Escherichia coli* BL21 (KRX #L3002, Promega), and clones were selected on solid medium containing ampicillin. After 3 h of induction at room temperature (RT) in the presence of 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and 0.1% rhamnose, cells were collected by centrifugation and maintained at  $-80$  °C.

The fusion proteins were purified under native conditions on GStrap FF columns (#17-5130-01, GE Healthcare, Madrid, Spain) or with HaloLink resin (#G1915, Promega). When necessary, retained fusion proteins were cleaved on the column with ProTEV protease (#V605A, Promega), and further purification was achieved by high-resolution ion-exchange separation (#780-0001Enrich Q, BioRad Laboratories, Madrid, Spain). Sequences were confirmed by automated capillary sequencing. Recombinant calmodulin (CaM, #208694, Merck-Millipore Iberica, Madrid, Spain) and BiP (#ab78432, Abcam, Cambridge, UK) were obtained from commercial sources.

#### 4.2. *In Vitro* Interactions between Recombinant Proteins: Pull-Down of Recombinant Proteins

Recombinant WT  $\sigma$ 1R and  $\sigma$ 1R E102Q (100 nM) were incubated with either Sepharose®4B (#17-0120-01, GE Healthcare; negative control) or with immobilized proteins, including the C-terminus of MOR1, the C0-C1-C2 region of the NMDAR NR1 subunit, the N- and C-terminal domains of TRP, HINT1, and BiP, which were covalently attached to NHS-activated Sepharose®4 Fast Flow (4FF, #17-0906-01, GE Healthcare) according to the manufacturer's instructions.

The interactions were studied in 300  $\mu$ L of buffer containing 50 mM Tris-HCl (pH 7.4) and 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in the presence of 2.5 mM  $\text{CaCl}_2$  and mixed by rotation for 30 min at RT. After incubation, the pellets were recovered by centrifugation, washed three times with 2.5 mM  $\text{CaCl}_2$ , solubilized in 2 $\times$  Laemmli buffer, and analyzed by western blotting.

The influence of added calcium on the association of WT  $\sigma$ 1R and  $\sigma$ 1R E102Q with the C0-C1-C2 region of the NR1 subunit, the C-terminus of TRPV1, and BiP was also evaluated. The recombinant  $\sigma$ 1R and its mutant (100 nM) were incubated either alone (negative control) or with immobilized proteins in 300  $\mu$ L of buffer containing 50 mM Tris-HCl (pH 7.4) and 0.2% CHAPS in the presence of increasing amounts of calcium chloride for 30 min at RT. This protocol was also carried out to assess the competition between CaM and high concentrations of WT  $\sigma$ 1R and the E102Q mutant for binding to the C0-C1-C2 region of the NR1 subunit.

To evaluate the effect of  $\sigma$ 1R ligands on the association between  $\sigma$ 1R E102Q and the NR1 subunit and between  $\sigma$ 1R E102Q and BiP, agarose-attached protein- $\sigma$ 1R E102Q complexes were incubated for an additional 30 min at RT with rotation in the presence of increasing concentrations of ligand in a final reaction volume of 300  $\mu$ L of 50 mM buffer containing Tris-HCl (pH 7.4), 2.5 mM  $\text{CaCl}_2$ , and 0.2% CHAPS. If an organic solvent was required for incorporation of the studied ligand, such as dimethyl sulfoxide (DMSO), the concentration of the solvent in the assay buffer remained below 1%. Agarose pellets containing bound proteins were obtained as described above. The following compounds were studied: progesterone (#P7556, Sigma-Aldrich Química, Madrid, Spain), pregnenolone sulfate (#P162, Sigma-Aldrich), S1RA (#16279, Cayman Chemical, Ann Arbor, MI, USA), and PPCC (#3870, Tocris Bioscience, Bristol, UK).

#### 4.3. Western Blotting

Unbound WT  $\sigma$ 1R and  $\sigma$ 1R E102Q proteins recovered by the procedure described above were resolved by SDS-PAGE on 4–12% Bis-Tris gels (#NP0341, Invitrogen, Fisher Scientific) with MES SDS running buffer (#NP0002, Invitrogen). The proteins were transferred onto 0.2  $\mu$ m polyvinylidene

difluoride (PVDF) membranes (#162-0176, BioRad) and probed overnight at 6 °C with anti- $\sigma$ 1R (#42-3300, Invitrogen, Fisher Scientific, Madrid, Spain) and anti-CaM (#05-173, Merck-Millipore) primary antibodies diluted in Tris-buffered saline (pH 7.7) (TBS) + 0.05% Tween 20 (TTBS). All primary antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies, which were visualized by chemiluminescence (#170-5061, BioRad) and imaged on an ImageQuant LAS 500 system (GE Healthcare). Single bands of the expected size were observed because all the assays were performed with recombinant proteins, and these bands were used for subsequent densitometric analysis. Accordingly, there were no bands on other areas of the blots, and these areas were routinely excluded from the analysis. The software automatically calculated the optimal exposure time for obtaining the strongest possible signal for each blot, allowing the bands to be accurately quantified. For each group of samples, the area of the strongest protein immunosignal was measured (average optical density of the pixels within the object area/mm<sup>2</sup>; AlphaEase FC software, Alpha Innotech, San Leandro, CA, USA). The gray values of the means were then normalized within the 8 bit/256 gray levels (256-computed value/computed value).

#### 4.4. Statistical Analyses

The western blot data were expressed as the change in signal relative to that of the control group, which was assigned an arbitrary value of 1. Statistical analyses were performed using the SigmaPlot/SigmaStat v. 14 package (Statistical Package for The Social Sciences (SPSS) software, Erkrath, Germany), and  $p < 0.05$  was considered to indicate significance. The data were analyzed using one-way ANOVA followed by the pairwise Holm–Sidak multiple comparison test.

**Supplementary Materials:** Supplementary materials can be found at <http://www.mdpi.com/1422-0067/21/19/7339/s1>. Figure S1. In vitro assay of the binding of  $\sigma$ 1R to different signaling proteins.

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#### Abbreviations

ALS	Amyotrophic lateral sclerosis
BiP	Binding immunoglobulin protein
CaM	Calmodulin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ER	Endoplasmic reticulum
FTLD	Frontotemporal lobar degeneration
GPCR	G-protein coupled receptor
HINT1	Histidine triad nucleotide-binding protein 1
IP3R3	Inositol 1,4,5-triphosphate receptor type 3
MAM	Mitochondria-associated ER membranes
MOR	Mu-opioid receptor
NMDAR	N-methyl-D-aspartate receptor

PPCC	2-[(4-Hydroxy-4-phenyl-1-piperidiny)methyl]-1-(4-methylphenyl)-cyclopropanecarboxylic acid
S1RA	4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl]morpholine
σ1R	Sigma 1 receptor
TRP	Transient receptor potential calcium channel
WT	Wild-type

## References

- Gundlach, A.L.; Largent, B.L.; Snyder, S.H. Autoradiographic localization of sigma receptor binding sites in guinea pig and rat central nervous system with (+)3H-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine. *J. Neurosci* **1986**, *6*, 1757–1770. [[CrossRef](#)] [[PubMed](#)]
- Largent, B.L.; Gundlach, A.L.; Snyder, S.H. Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)-[3H]SKF 10,047, (+)-[3H]-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine and [3H]-1-[1-(2-thienyl)cyclohexyl]piperidine. *J. Pharm. Exp.* **1986**, *238*, 739–748.
- Zukin, S.R.; Tempel, A.; Gardner, E.L.; Zukin, R.S. Interaction of [3H](-)-SKF-10,047 with brain sigma receptors: Characterization and autoradiographic visualization. *J. Neurochem* **1986**, *46*, 1032–1041. [[CrossRef](#)] [[PubMed](#)]
- Walker, J.M.; Bowen, W.D.; Goldstein, S.R.; Roberts, A.H.; Patrick, S.L.; Hohmann, A.G.; DeCosta, B. Autoradiographic distribution of [3H](+)-pentazocine and [3H]1,3-di-o-tolylguanidine (DTG) binding sites in guinea pig brain: A comparative study. *Brain Res.* **1992**, *581*, 33–38. [[CrossRef](#)]
- Kim, F.J.; Kovalyshyn, I.; Burgman, M.; Neilan, C.; Chien, C.C.; Pasternak, G.W. Sigma 1 receptor modulation of G-protein-coupled receptor signaling: Potentiation of opioid transduction independent from receptor binding. *Mol. Pharm.* **2010**, *77*, 695–703. [[CrossRef](#)] [[PubMed](#)]
- Navarro, G.; Moreno, E.; Aymerich, M.; Marcellino, D.; McCormick, P.J.; Mallol, J.; Cortes, A.; Casado, V.; Canela, E.I.; Ortiz, J.; et al. Direct involvement of sigma-1 receptors in the dopamine D1 receptor-mediated effects of cocaine. *Proc. Natl Acad Sci USA* **2010**, *107*, 18676–18681. [[CrossRef](#)]
- Sánchez-Blázquez, P.; Rodríguez-Muñoz, M.; Herrero-Labrador, R.; Burgueño, J.; Zamanillo, D.; Garzón, J. The calcium-sensitive Sigma-1 receptor prevents cannabinoids from provoking glutamate NMDA receptor hypofunction: Implications in antinociception and psychotic diseases. *Int J. Neuropsychopharmacol* **2014**, *17*, 1943–1955. [[CrossRef](#)]
- Hayashi, T.; Su, T.P. Sigma-1 receptors (sigma(1) binding sites) form raft-like microdomains and target lipid droplets on the endoplasmic reticulum: Roles in endoplasmic reticulum lipid compartmentalization and export. *J. Pharm. Exp.* **2003**, *306*, 718–725. [[CrossRef](#)]
- Pal, A.; Fontanilla, D.; Gopalakrishnan, A.; Chae, Y.K.; Markley, J.L.; Ruoho, A.E. The sigma-1 receptor protects against cellular oxidative stress and activates antioxidant response elements. *Eur. J. Pharm.* **2012**, *682*, 12–20. [[CrossRef](#)]
- Tsai, S.Y.; Chuang, J.Y.; Tsai, M.S.; Wang, X.F.; Xi, Z.X.; Hung, J.J.; Chang, W.C.; Bonci, A.; Su, T.P. Sigma-1 receptor mediates cocaine-induced transcriptional regulation by recruiting chromatin-remodeling factors at the nuclear envelope. *Proc Natl Acad Sci USA* **2015**, *112*, E6562–E6570. [[CrossRef](#)]
- Hayashi, T.; Su, T.P. Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. *Cell* **2007**, *131*, 596–610. [[CrossRef](#)] [[PubMed](#)]
- Hayashi, T.; Su, T.P. The potential role of sigma-1 receptors in lipid transport and lipid raft reconstitution in the brain: Implication for drug abuse. *Life Sci* **2005**, *77*, 1612–1624. [[CrossRef](#)] [[PubMed](#)]
- Maurice, T.; Su, T.P. The pharmacology of sigma-1 receptors. *Pharmacology* **2009**, *124*, 195–206. [[CrossRef](#)] [[PubMed](#)]
- Mei, J.; Pasternak, G.W. Molecular cloning and pharmacological characterization of the rat sigma1 receptor. *Biochem. Pharm.* **2001**, *62*, 349–355. [[CrossRef](#)]
- Sabino, V.; Cottone, P.; Parylak, S.L.; Steardo, L.; Zorrilla, E.P. Sigma-1 receptor knockout mice display a depressive-like phenotype. *Behav Brain Res.* **2009**, *198*, 472–476. [[CrossRef](#)]
- Ishiguro, H.; Ohtsuki, T.; Toru, M.; Itokawa, M.; Aoki, J.; Shibuya, H.; Kurumaji, A.; Okubo, Y.; Iwawaki, A.; Ota, K.; et al. Association between polymorphisms in the type 1 sigma receptor gene and schizophrenia. *Neurosci Lett* **1998**, *257*, 45–48. [[CrossRef](#)]

17. Sánchez-Blázquez, P.; Pozo-Rodrigálvarez, A.; Merlos, M.; Garzón, J. The Sigma-1 Receptor Antagonist, S1RA, Reduces Stroke Damage, Ameliorates Post-Stroke Neurological Deficits and Suppresses the Overexpression of MMP-9. *Mol. Neurobiol.* **2018**, *55*, 4940–4951. [[CrossRef](#)]
18. Wang, J.; Cui, X.; Roon, P.; Saul, A.; Smith, S.B. The Role of Sigma1R in Mammalian Retina. *Adv. Exp. Med. Biol.* **2017**, *964*, 267–284. [[CrossRef](#)]
19. Mavlyutov, T.A.; Nickells, R.W.; Guo, L.W. Accelerated retinal ganglion cell death in mice deficient in the Sigma-1 receptor. *Mol. Vis.* **2011**, *17*, 1034–1043.
20. Villard, V.; Espallergues, J.; Keller, E.; Vamvakides, A.; Maurice, T. Anti-amnesic and neuroprotective potentials of the mixed muscarinic receptor/sigma 1 (sigma1) ligand ANAVEX2-73, a novel aminotetrahydrofuran derivative. *J. Psychopharmacol.* **2011**, *25*, 1101–1117. [[CrossRef](#)]
21. Francardo, V.; Bez, F.; Wieloch, T.; Nissbrandt, H.; Ruscher, K.; Cenci, M.A. Pharmacological stimulation of sigma-1 receptors has neurorestorative effects in experimental parkinsonism. *Brain* **2014**, *137*, 1998–2014. [[CrossRef](#)] [[PubMed](#)]
22. Hyrskyluoto, A.; Pulli, I.; Tornqvist, K.; Ho, T.H.; Korhonen, L.; Lindholm, D. Sigma-1 receptor agonist PRE084 is protective against mutant huntingtin-induced cell degeneration: Involvement of calpastatin and the NF-kappaB pathway. *Cell. Death Dis.* **2013**, *4*, e646. [[CrossRef](#)] [[PubMed](#)]
23. Luty, A.A.; Kwok, J.B.; Dobson-Stone, C.; Loy, C.T.; Coupland, K.G.; Karlstrom, H.; Sobow, T.; Tchorzewska, J.; Maruszak, A.; Barcikowska, M.; et al. Sigma nonopioid intracellular receptor 1 mutations cause frontotemporal lobar degeneration-motor neuron disease. *Ann. Neurol.* **2010**, *68*, 639–649. [[CrossRef](#)] [[PubMed](#)]
24. Li, X.; Hu, Z.; Liu, L.; Xie, Y.; Zhan, Y.; Zi, X.; Wang, J.; Wu, L.; Xia, K.; Tang, B.; et al. A SIGMAR1 splice-site mutation causes distal hereditary motor neuropathy. *Neurology* **2015**, *84*, 2430–2437. [[CrossRef](#)] [[PubMed](#)]
25. Al-Saif, A.; Al-Mohanna, F.; Bohlega, S. A mutation in sigma-1 receptor causes juvenile amyotrophic lateral sclerosis. *Ann. Neurol.* **2011**, *70*, 913–919. [[CrossRef](#)] [[PubMed](#)]
26. Watanabe, S.; Ilieva, H.; Tamada, H.; Nomura, H.; Komine, O.; Endo, F.; Jin, S.; Mancias, P.; Kiyama, H.; Yamanaka, K. Mitochondria-associated membrane collapse is a common pathomechanism in SIGMAR1- and SOD1-linked ALS. *Embo Mol. Med.* **2016**, *8*, 1421–1437. [[CrossRef](#)] [[PubMed](#)]
27. Mavlyutov, T.A.; Epstein, M.L.; Andersen, K.A.; Ziskind-Conhaim, L.; Ruoho, A.E. The sigma-1 receptor is enriched in postsynaptic sites of C-terminals in mouse motoneurons. An anatomical and behavioral study. *Neuroscience* **2010**, *167*, 247–255. [[CrossRef](#)]
28. Bernard-Marissal, N.; Medard, J.J.; Azzedine, H.; Chrast, R. Dysfunction in endoplasmic reticulum-mitochondria crosstalk underlies SIGMAR1 loss of function mediated motor neuron degeneration. *Brain* **2015**, *138*, 875–890. [[CrossRef](#)]
29. Schmidt, H.R.; Zheng, S.; Gurpinar, E.; Koehl, A.; Manglik, A.; Kruse, A.C. Crystal structure of the human sigma1 receptor. *Nature* **2016**, *532*, 527–530. [[CrossRef](#)]
30. Dreser, A.; Vollrath, J.T.; Sechi, A.; Johann, S.; Roos, A.; Yamoah, A.; Katona, I.; Bohlega, S.; Wiemuth, D.; Tian, Y.; et al. The ALS-linked E102Q mutation in Sigma receptor-1 leads to ER stress-mediated defects in protein homeostasis and dysregulation of RNA-binding proteins. *Cell Death Differ.* **2017**, *24*, 1655–1671. [[CrossRef](#)]
31. Rodríguez-Muñoz, M.; Sánchez-Blázquez, P.; Herrero-Labrador, R.; Martínez-Murillo, R.; Merlos, M.; Vela, J.M.; Garzón, J. The sigma1 receptor engages the redox-regulated HINT1 protein to bring opioid analgesia under NMDA receptor negative control. *Antioxid. Redox Signal.* **2015**, *22*, 799–818. [[CrossRef](#)] [[PubMed](#)]
32. Rodríguez-Muñoz, M.; Cortés-Montero, E.; Pozo-Rodrigálvarez, A.; Sánchez-Blázquez, P.; Garzón-Niño, J. The ON:OFF switch, sigma1R-HINT1 protein, controls GPCR-NMDA receptor cross-regulation: Implications in neurological disorders. *Oncotarget* **2015**, *6*, 35458–35477. [[CrossRef](#)] [[PubMed](#)]
33. Cortés-Montero, E.; Sánchez-Blázquez, P.; Onetti, Y.; Merlos, M.; Garzón, J. Ligands Exert Biased Activity to Regulate Sigma 1 Receptor Interactions With Cationic TRPA1, TRPV1, and TRPM8 Channels. *Front. Pharm.* **2019**, *10*, 634. [[CrossRef](#)] [[PubMed](#)]
34. Lu, H.; Le, W.D.; Xie, Y.Y.; Wang, X.P. Current Therapy of Drugs in Amyotrophic Lateral Sclerosis. *Curr. Neuropharmacol.* **2016**, *14*, 314–321. [[CrossRef](#)] [[PubMed](#)]

35. Rodríguez-Muñoz, M.; Sánchez-Blázquez, P.; Garzón, J. Fenfluramine diminishes NMDA receptor-mediated seizures via its mixed activity at serotonin 5HT<sub>2A</sub> and type 1 sigma receptors. *Oncotarget* **2018**, *9*, 23373–23389. [[CrossRef](#)] [[PubMed](#)]
36. Hayashi, T.; Su, T.P. Regulating ankyrin dynamics: Roles of sigma-1 receptors. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 491–496. [[CrossRef](#)] [[PubMed](#)]
37. Ehlers, M.D.; Zhang, S.; Bernhardt, J.P.; Huganir, R.L. Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **1996**, *84*, 745–755. [[CrossRef](#)]
38. Wong, A.Y.; Hristova, E.; Ahlskog, N.; Tasse, L.A.; Ngsee, J.K.; Chudalayandi, P.; Bergeron, R. Aberrant Subcellular Dynamics of Sigma-1 Receptor Mutants Underlying Neuromuscular Diseases. *Mol. Pharm.* **2016**, *90*, 238–253. [[CrossRef](#)]
39. Lipton, S.A. Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. *Nat. Rev. Drug Discov.* **2006**, *5*, 160–170. [[CrossRef](#)]
40. Cortés-Montero, E.; Rodríguez-Muñoz, M.; Sánchez-Blázquez, P.; Garzón, J. The Axonal Motor Neuropathy-Related HINT1 Protein Is a Zinc- and Calmodulin-Regulated Cysteine SUMO Protease. *Antioxid. Redox Signal.* **2019**, *31*, 503–520. [[CrossRef](#)]
41. Zimon, M.; Baets, J.; Almeida-Souza, L.; De, V.E.; Nikodinovic, J.; Parman, Y.; Battaloglu, E.; Matur, Z.; Guergueltcheva, V.; Tournev, I.; et al. Loss-of-function mutations in HINT1 cause axonal neuropathy with neuromyotonia. *Nat. Genet.* **2012**, *44*, 1080–1083. [[CrossRef](#)] [[PubMed](#)]
42. Weiske, J.; Huber, O. The histidine triad protein Hint1 interacts with Pontin and Reptin and inhibits TCF-beta-catenin-mediated transcription. *J. Cell Sci.* **2005**, *118*, 3117–3129. [[CrossRef](#)] [[PubMed](#)]
43. Leamey, C.A.; Sawatari, A. Teneurins: Mediators of Complex Neural Circuit Assembly in Mammals. *Front. Neurosci.* **2019**, *13*, 580. [[CrossRef](#)] [[PubMed](#)]
44. Mosca, T.J.; Hong, W.; Dani, V.S.; Favaloro, V.; Luo, L. Trans-synaptic Teneurin signalling in neuromuscular synapse organization and target choice. *Nature* **2012**, *484*, 237–241. [[CrossRef](#)]
45. Suzuki, N.; Fukushi, M.; Kosaki, K.; Doyle, A.D.; de Vega, S.; Yoshizaki, K.; Akazawa, C.; Arikawa-Hirasawa, E.; Yamada, Y. Teneurin-4 is a novel regulator of oligodendrocyte differentiation and myelination of small-diameter axons in the CNS. *J. Neurosci.* **2012**, *32*, 11586–11599. [[CrossRef](#)] [[PubMed](#)]
46. Almendra, L.; Laranjeira, F.; Fernandez-Marmiesse, A.; Negrão, L. SIGMAR1 gene mutation causing Distal Hereditary Motor Neuropathy in a Portuguese family. *Acta Myol.* **2018**, *37*, 2–4. [[PubMed](#)]
47. Gregianin, E.; Pallafacchina, G.; Zanin, S.; Crippa, V.; Rusmini, P.; Poletti, A.; Fang, M.; Li, Z.; Diano, L.; Petrucci, A.; et al. Loss-of-function mutations in the SIGMAR1 gene cause distal hereditary motor neuropathy by impairing ER-mitochondria tethering and Ca<sup>2+</sup> signalling. *Hum. Mol. Genet.* **2016**, *25*, 3741–3753. [[CrossRef](#)]
48. Ullah, M.I.; Ahmad, A.; Raza, S.I.; Amar, A.; Ali, A.; Bhatti, A.; John, P.; Mohyuddin, A.; Ahmad, W.; Hassan, M.J. In silico analysis of SIGMAR1 variant (rs4879809) segregating in a consanguineous Pakistani family showing amyotrophic lateral sclerosis without frontotemporal lobar dementia. *Neurogenetics* **2015**, *16*, 299–306. [[CrossRef](#)]
49. Fukunaga, K.; Shinoda, Y.; Tagashira, H. The role of SIGMAR1 gene mutation and mitochondrial dysfunction in amyotrophic lateral sclerosis. *J. Pharm. Sci.* **2015**, *127*, 36–41. [[CrossRef](#)]
50. Ryskamp, D.A.; Zhemkov, V.; Bezprozvanny, I. Mutational Analysis of Sigma-1 Receptor's Role in Synaptic Stability. *Front. Neurosci.* **2019**, *13*, 1012. [[CrossRef](#)]
51. Su, T.P.; Hayashi, T.; Maurice, T.; Buch, S.; Ruoho, A.E. The sigma-1 receptor chaperone as an inter-organelle signaling modulator. *Trends Pharm. Sci.* **2010**, *31*, 557–566. [[CrossRef](#)] [[PubMed](#)]
52. Abramyan, A.M.; Yano, H.; Xu, M.; Liu, L.; Naing, S.; Fant, A.D.; Shi, L. The Glu102 mutation disrupts higher-order oligomerization of the sigma 1 receptor. *Comput. Struct. Biotechnol. J.* **2020**, *18*, 199–206. [[CrossRef](#)] [[PubMed](#)]

