SIGMA-1 RECEPTOR (σ – 1R) ACTIVATION AND MODULATION OF NMDA RECEPTOR SURFACE EXPRESSION

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ABSTRACT

The sigma-1 receptors (σ-1Rs) are endoplasmic reticulum (ER) resident proteins shown to have chaperone-like functions, and are widely distributed throughout the central nervous system (CNS). They reside at a specialized membrane called mitochondria-associated ER-membrane (MAM) and can modulate numerous voltage- and ligand-gated ion channels. One of these channels is the *N*-methyl-D-aspartate receptor (NMDAR), and σ-1R ligands are able to enhance the potentiation of NMDARs, but the mechanism involved remains poorly understood. Using various biochemical techniques, we show that 90 min following an *i.p.* injection of σ-1R agonists ((+)-SKF 10,047 (SKF), (+)-Pentazocine (PTZ), or PRE-084 (PRE), there is an increase in the expression of GluN2-containing NMDARs in the rat hippocampus. These results suggest that σ-1R activation is able to enhance NMDAR function by modulating protein expression levels both in the cytosol and on the cell surface. This suggests that σ-1Rs could be excellent therapeutic targets for many neurological disorders, and for the development of novel antipsychotics.

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LIST OF ABBREVIATIONS

σ-1R	Sigma-1 Receptor
σ-2R	Sigma-2 Receptor
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Action Potential
AP-1	Activator protein 1
AP-2	Activator protein 2
ATD	Amino Terminal Domain
BiP	Binding Immunoglobulin Protein
CNS	Central Nervous System
CTD	C-terminal Domain
EPSC	Excitatory Post Synaptic Current
EPSP	Excitatory Post Synaptic Potential
ER	Endoplasmic Reticulum
FTLD	Frontotemporal Lobar Degeneration
GABA	ϒ-Aminobutyric Acid
GATA-1	Globin transcription factor 1
i.p.	Intraperitoneal
IP_3R	1-4-5-triphosphate receptor
IPSC	Inhibitory Post Synaptic Current
IPSP	Inhibitory Post Synaptic Potential
LBD	Ligand Binding Domain
LTD	Long Term Depression
LTP	Long Term Potentiation
MAM	Mitochondria Associated ER Membrane
MND	Motor Neuron Degeneration
NF-1	Nuclear factor 1
NMDAR	*N*-methyl-D-aspartate receptor

PRE-084	1-phenyl-2-(4-morpholinyl)ethyl ester-cyclohexanecarboxylic acid
PSD	Post Synaptic Density
PTZ	Pentazocine
SAP-102	Synapse Associated Protein 102
SKF	SKF-10,047
	N-allylnormetazocine
WB	Western Blot

Sigma-1 Receptors (σ-1Rs)

History

The concept of sigma receptors (σ-Rs) arose almost 40 years ago in a pharmacological study which postulated the existence of three different types of opioid receptors (μ, κ, and σ receptor) accounting for the psyhotomimetic effects produced by opioid compounds such as morphine, ketacyclazocine, and SKF10,047 (SKF) respectively (Martin *et al.*, 1976; reviewed by Crottes *et al.*, 2013). In contrast to the morphine induced analgesia, benzomorphans like SKF and pentazocine (PTZ) can cause psychotomimesis in the dog (Martin *et al.*, 1976). Following its discovery the receptor was initially called an opioid receptor because the effect of SKF was reported to be antagonized by the universal opioid antagonist, naloxone. In a later study, a protein with nanomolar affinity for SKF but no affinity for the opioid receptor antagonist, naloxone, was identified and termed the sigma/opioid receptors (Su *et al.*, 1982), which was not the same receptor identified by Martin *et al.* (1976) because the latter was insensitive to naloxone. These experiments were later repeated and it was found that the "psychotomimetic" effect induced by SKF was not blocked by another potent analog of naloxone, naltrexone (Vaupel, 1983; Su *et al.*, 2009). This receptor had higher affinity for dextrorotatory benzomorphans like (+)-SKF and (+)-PTZ than for levorotatory isomers (*e.g.* (−)-SKF, (−)-PTZ). The stereospecificity is opposite to what is seen with opioid receptor subtypes in binding assays or behavioral tests, which further supports that the SKF binding protein initially described by Martin (1976) is not an opioid receptor after all. The protein identified by

Su was later termed “sigma receptor” to distinguish it from receptors in the opioid receptor family.

Since the discovery of the σ receptor, many σ receptor ligands have been identified (Table 1) and two σ receptor subtypes have been identified based on their opposite enantioselectivity for benzomorphans (Martin *et al.,* 1976). The two σ receptor subtypes are the σ-1R and the σ-2R (Martin *et al.,* 1976; Hellewell *et al.,* 1994), and a recent study postulated the existence of a third σ receptor (Booth *et al.,* 1999). One of the few known differences between σ-1Rs and σ-2Rs, is that σ-1Rs display stereospecificity towards (+)-isomers of benzomorphans (*e.g.* (+)-PTZ, (+)-SKF), while σ-2Rs are more selective towards (−)-isomers ((−)-PTZ, (−)-SKF). The σ-1Rs were initially cloned in 1996, while the σ-2R remains elusive and little is known about it.

σ-1R Molecular and Gene Structure

The σ-1R is a 223 amino acid protein with a molecular weight of approximately 24 kDa. It contains three hydrophobic domains, two transmembrane regions and two steroid binding domains (Fig 1). It shares no sequence homology to any other known mammalian proteins, although the σ-1 receptor amino acid sequence does share sequence homology with ERG2, a fungal gene encoding sterol isomerase (Hanner *et al.,* 1996; reviewed by Hayashi & Su, 2005; Ishikawa & Hashimoto, 2009). In a 218 amino acid overlap, these sequences are 30.3% identical and 66.4% similar with the σ-1 receptor binding site, suggesting that the protein forming the σ-1 receptor binding site represents the mammalian counterpart of fungal sterol C7–C8 isomerase, although it doesn’t exhibit

such activity (Hanner *et al.,* 1996). The amino acid sequence of the σ-1R exhibits more than 90% identity between different mammalian species, and since the first cloning of guinea pig σ-1Rs (Hanner *et al.,* 1996), σ-1R genes have been cloned from several mammalian cDNA libraries, including human, mouse and rat (Kekuda *et al.,* 1996; Seth *et al.,* 1998; Seth *et al.,* 1997).

The entire sequence of the σ-1R gene has been extensively examined. The human σ-1R gene is approximately 7 kbp long and is encoded by four exons (207, 201, 93, and 1132 bp in size), with three introns (126, 130, and 1250 bp in size) (Prasad *et al.,* 1998). Exon 1 contains the 5' untranslated region and part of the protein coding sequence while exon 2 codes for the initially proposed single transmembrane domain (Prasad *et al.,* 1998). It has since been shown that the σ-1R contains two transmembrane regions, and it has been suggested that the hydrophobic region in the middle of the protein could be the transmembrane domain (Aydar *et al.,* 2002; summarized in Hayashi & Su, 2005). Exon 3 is the shortest, and exon 4 contains the 3' untranslated region and part of the protein coding sequence, as well as the polyadenylation signal (AATAAA) (Prasad *et al.,* 1998).

The σ-1R gene does not contain a TATA-box, but contains a CCAATC box in the reverse complement and several GC boxes that are recognition sites for the transcription factor SP1. Such GC repeats and the absence of a TATA box are known to play a role in the binding of various transcription factors. The gene has been shown to be located on human chromosome 9, band p13 – a region known to be associated with many psychiatric disorders (Prasad *et al.,* 1998). The amino acid sequence of the σ-1R contains a double

arginine at the N-terminus, which has been speculated to be an ER retention signal (Hanner *et al.,* 1996), which is in accordance with the current hypothesis that the σ-1R is an ER resident protein.

The mouse σ-1R has been shown to contain consensus binding sites for several transcription proteins including: transcription factor activator protein 1 and 2 (AP-1 and AP-2), nuclear factor 1 (NF-1), and globin transcription factor 1 (GATA-1), within 1 kbp upstream of the transcription start site, as well as several binding sites for steroid receptors in the 5'-flanking region (Seth *et al.,* 1997). The two transmembrane domains and the two steroid binding domains of the σ-1R form a pocket that is the binding site for cholesterol, steroids, sphingolipids (Palmer *et al.,* 2007; Fontanilla *et al.,* 2008). Many synthetic and natural compounds (sigma ligands) from different classes such as opioids, antipsychotics, psychotostimulants, alkaloids, or antidepressants have also been shown to bind to σ-1R (Pal *et al.,* 2008; Maurice & Su, 2009).

Distribution

System Wide

At the anatomical level, σ-1Rs are widely distributed in both the central and peripheral nervous system, where they regulate neurite growth, potassium channel function, memory, drug addiction, and IP3R-mediated Ca^{2+} signaling, as well as in peripheral organs like the liver and kidney. Using [^{35}S]UTP-labeled antisense riboprobes, Kitaichi *et al.* (2000) looked at the expression of σ-1Rs in the rodent central nervous system (CNS).

The highest levels of hybridization were in the densely packed granular lamina and the glomerular cell layers of the olfactory bulb. Moderate σ-1R mRNA levels were found in the hippocampus, and in the adult mouse and guinea pig brain the medial habenula were particularly enriched with σ-1R mRNA. Lower levels of σ-1R mRNA were also observed in various nuclei such as the arcuate, paraventricular and ventromedial hypothalamic as well as low to moderate levels in the spleen. Kitaichi *et al.* (2000) also looked at σ-1R expression in the human CNS, using post mortem tissue, and they found the highest hybridization signal to be in the stratum granulosum of the dentate gyrus as well as moderate levels of σ-1R mRNA in the stratum pyramidale. The presence of moderate levels of σ-1R gene expression in the hippocampal formation and other limbic areas is in accordance with the alleged role of the σ-1R in the modulation of cognitive processes such as learning and memory.

Subcellular

Studies have suggested that the σ-1R is located in the cytoplasm (Hayashi & Su, 2001), on the cell surface (Lupardus *et al.*, 2000), or in both places (Morin-Surun *et al.,* 1999). At the mitochondria-associated ER-membrane (MAM), σ-1 receptors reside in the ceramide-enriched microdomains where they appear to be bound to ceramide and ER chaperone proteins (Hayashi & Su, 2010). It has also been reported that σ-1Rs translocate from the cytoplasmic membrane to the vicinity of the cell membrane following activation (Morin-Surun *et al.,* 1999; Hayashi & Su, 2001).

Early studies combining the subcellular fractionation with the radioligand binding assay indicated that σ-1Rs are enriched at the microsomal membrane (Cagnotto *et al.,* 1994; McCann & Su, 1990). Although data suggests that σ-1Rs are localized in the ER membrane, immunocytochemical studies have shown detailed subcellular distribution of σ-1Rs in the cytoplasmic region of cell bodies in most cells (Alonso *et al.,* 2000; Dussossoy *et al.,* 1999; Hayashi & Su, 2003; Jbilo *et al.,* 1997). Immunofluorescence studies have also shown that σ-1R antibodies predominantly stain cytoplasmic areas in neuronal and retinal cells as well (Alonso *et al.,* 2000; Hayashi & Su, 2003; Morin-Surun *et al.,* 1999; Shamsul *et al.,* 2001), and an electron microscopy study of rat brain slices indicated that σ-1Rs were mostly associated with the perikarya and dendrites of neurons (Alonso *et al.,* 2000). At the level of synaptic contacts, σ-1R was associated with the postsynaptic density, whereas the presynaptic axons lacked any association (Alonso *et al.,* 2000). These studies show that σ-1Rs localize primarily on the ER of the cell body and show a post-synaptic distribution in neurons in the brain.

A few recent studies indicate that ER lipid rafts may play roles in the segregation of ER proteins and in cellular differentiation (Hayashi & Su 2004; Muniz *et al.,* 2001). In recent years it has also been found that σ-1Rs are present on nuclear envelopes and ER networks (Hayashi & Su 2003a; Hayashi & Su 2003b). It has moreover been shown that σ-1Rs are highly enriched at lipid rafts, consisting of ceramide, sphingolipids and cholesterol, and have been shown to regulate a variety of cellular functions like protein sorting and clustering of scaffold proteins (Anderson, 1993; Simons & Ikonen, 1997).

Since σ-1Rs are widely distributed not only within the CNS but also within the subcellular environment, many studies have looked at its involvement in neurological diseases. There is compelling evidence for the involvement of σ-1Rs in major depressive disorder like Alzheimer's disease (AD), schizophrenia, drug addiction and stroke, as well as important role of σ-1R mutations and their potential involvement in amyotrophic lateral sclerosis (ALS) and other neurodegenerative disorders.

Function

σ-1R Function

At the MAM, σ-1Rs reside in the ceramide-enriched microdomains where they have been shown to bind ceramide and be associated with lipid rafts (Hayashi & Su 2010). It has been proposed that lipid rafts function as platforms for the attachment of proteins, when membranes are moved around inside the cell and during signal transduction (reviewed by Simons & Ikonen). They have also been shown to bind another ER chaperone protein, binding immunoglobulin protein, or BiP. BiP is a major ER chaperone protein critical for protein quality of the ER, and is obligatory for early embryonic development (reviewed by Wang *et al*., 2009). Mouse models reveal that BiP is involved in the maturation and secretion of neuronal factors for proper neural migration, and could be involved in neuroprotection (reviewed by Wang *et al*., 2009). BiP has also been termed the master regulator of the unfolded protein response (UPR), which is a series of adaptive responses in reaction to ER stress. The exact locus of binding between σ-1Rs, BiP, or ceramide is currently unknown. The binding of σ-1Rs to BiP normally prevents the σ-1Rs from translocation (Hayashi & Su 2003; Hayashi & Su 2007). A decrease in Ca^{2+} at the ER

due to ER stress, or activation of the σ-1R *via* agonists, causes the σ-1R to dissociate from the BiP complex, causing it to become an active chaperone (Ishikawa & Hashimoto, 2009), and will be explained in the following section.

It has also been shown that σ-1Rs are able to potentiate NMDA-induced neuronal firing in the CA3 region of the hippocampus and that this potentiation is blocked by the σ-1R specific antagonist, haloperidol (Monnet *et al.,* 1990). The same group also showed that σ-1R agonists potentiate the NMDA-induced Ca^{2+} influx from the extracellular space, and increase in intracellular Ca^{2+} concentration is blocked by another σ-1R specific antagonist, NE-100 (Monnet *et al.,* 2003). The σ-1Rs have also been implicated in the formation of dendritic spines in hippocampal neurons, as a reduction of extensions and branching of dendrites was observed in neurons transfected with σ-1R siRNA (Tsai *et al.,* 2009). The same study also suggests that σ-1R knockdown inhibits synapse formation as there were less functional synapses formed in neurons transfected with the σ-1R siRNA when compared to control (Tsai *et al.,* 2009). One of the most striking feature of σ-1Rs, is their ability to interact with numerous target proteins, many of which include ion channels, and modulate their functions. Some of these ion channels include, Ca^{2+} channels, potassium channels, and sodium channels.

σ-1R Regulation of Ion Channels

Ca^{2+} channels

The most prominent and the most explored molecular action of σ-1Rs is the receptor's interactions with ion channels. The ability of σ-1Rs to modulate Ca^{2+} channels has been

shown, but it is unknown whether this is *via* a direct or indirect action. A diverse range of cellular processes, such as gene transcription, ion channel function, muscle contraction, and cell proliferation are controlled by Ca^{2+} influx and homeostasis. Calcium channels are able to control the flux of calcium from extracellular to intracellular compartments, and this may regulate neurotransmitter release at the synaptic level. Calcium has also been shown to be able to act as a second messenger to trigger specific signaling pathways, and due to this, it needs to be very tightly regulated.

As mentioned earlier, the dissociation of σ-1R from its binding partner, BiP, frees the σ-1R, which then binds to the inositol 1-4-5-triphosphate receptor (IP_3R), which is normally very unstable and easily degraded. The binding of the σ-1R stabilizes the IP_3R, and as a result of this stabilization, Ca^{2+} is able to flow into the mitochondria via voltage-dependent ion channels (Hayashi and Su, 2007; reviewed by Ishikawa & Hashimoto, 2009). Although the exact interaction remains to be clarified in neurons, in NG-108 cells σ-1Rs form a trimeric complex with IP_3Rs and akryrin B, a cytoskeletal adaptor protein (Hayashi & Su 2001). Activation of σ-1R *via* agonists such as cocaine and pregnenolone sulfate, caused the dissociation of an ankyrin B isoform from type 3 IP_3Rs, an effect blocked by σ-1R antagonists (Hayashi & Su, 2001). However, σ-1Rs do not chaperone the IP_3Rs at the general ER network, since they reside specifically at the MAM, and therefore do not affect the dynamic cytosolic Ca^{2+} concentration (Hayashi & Su, 2001; Hayashi & Su, 2007).

Potassium Channels

In cultured frog pituitary melanotrope cells, the σ-1R ligands igmesine and PTZ dose-dependently decreased the transient outward potassium current, an effect blocked by the σ-1R antagonist NE-100 (Soriani *et al.,* 1999). The sustained, but no the transient outward potassium current was also decreased by σ-1R agonists (Soriani *et al.,* 1999b). Lupardus *et al.* (2000) found that σ-1R ligands inhibited the potassium channels in a GTP- and ATP-dependent manner, and that this interaction between σ-1Rs and potassium channels perhaps occurs within close proximity of one another. The σ-1Rs were immunoprecipitated with Kv1.4 potassium channels suggesting that the σ-1R acts as a ligand-regulated auxiliary potassium channel subunit (Aydar *et al.,* 2002, reviewed by Kourrich *et al.,* 2012).

Sodium Channels

Using rat medial prefrontal cortex slices, Cheng *et al.* (2008) found that the σ-1R antagonist DHEA sulfate inhibited persistent sodium currents, an effect that was improved by G_i protein and protein kinase C inhibitors (Cheng *et al.,* 2008,). Other σ-1R agonist mimicked the DHEA sulfate effect, and this was blocked by σ-1R antagonist (Cheng *et al.,* 2008). It has been shown that σ-1Rs are able to inhibit the volume-regulated chloride channels and that σ-1R ligands further activate the channel-inhibiting activity of σ-1Rs (Renaudo *et al.,* 2007).

Knockout Model

Following the cloning of the σ-1R, it was important to generate a knockout model in order to be able to further elucidate the functional roles of the σ-1R. The σ-1R knock out mice were generated by Langa *et al.* (2003) by disrupting the σ-1R gene in mouse embryonic stem cells using a replacement vector approach resulting in the replacement of 1907 nucleotides of mouse σ-1R genomic sequences (Langa *et al.,* 2003). Embryonic stem cell clones harboring the desired homologous recombination event were identified, and used to generate mouse chimeras by morula aggregation procedures.

The lack of σ-1R gene in the mutant animals was evaluated and σ-1R signals could not be detected in protein extracts prepared from mutant animals, while they were clearly visible in the wild-type animals (Langa *et al.,* 2003). The mutant σ-1R knockout mice exhibit an apparently normal phenotype, and show no gross morphological defects, and are not obviously distinguishable from their wild-type or heterozygous littermates (Langa *et al.,* 2003). Differences between wild-type and mutant pups at similar ages were not statistically significant. What is also important to note is that both the male and female mutant σ-1R knockout mice are fertile and can be used to establish subsequent homozygous mutant mouse colonies (Langa *et al.,* 2003). However, it has been shown that σ-1R knockout mice have impaired neurogenesis in the dentate gyrus through the downregulation of NMDA receptors (Sha *et al.,* 2013). Increased immobility in the forced swimming test, which is a depressive-like phenotype, while exhibiting normal anxiety-like behavior and locomotor activity is also present in σ-1R knockout mice (Sabino *et al.,* 2009).

σ-1R Mutations and Involvement in Diseases

Mutations

Frontotemporal lobar degeneration (FTLD) is the third most common cause of dementia, after AD and dementia with Lewy bodies, and it is the most common cause of dementia under the age of 65 (Panegyres *et al.*, 2007). The spectrum of FTLD phenotypes has been shown to include co-occurrence FTLD with motor neuron disease (MND). A nonpolymorphic mutation in the 3' untranslated region of the σ-1R gene in individuals affected by both FTLD and MND has been found (Luty *et al.*, 2010). This mutation increased overall σ-1R gene expression and resulted in elevated levels of σ-1R transcript or protein in lymphocyte or brain tissue. The authors concluded that the σ-1R gene is a causative gene for familial FTLD-MND with a unique neuropathology that differs from other FTLD and MND cases, suggesting that σ-1R drugs could serve as potential treatments for this pathophysiology.

In a recent study, a σ-1R mutation was associated with juvenile amyotrophic sclerosis (ALS) (Al-Saif *et al.*, 2011). ALS is a progressive neurodegenerative disorder that affects both upper and lower motor neurons and leads to death from respiratory failure. While 90% of cases do not have a family history of the disease, 10% have more than one affected family member, and this is known as familial-ALS. This study looked at individuals from the ALS002 families, which were diagnosed with juvenile ALS. They found a single shared homozygosity region in the affected individuals that was not shared by their unaffected siblings. This relatively small region spans approximately 120 kbp on chromosome 9p13.3 and was shown to overlap the ALS-FTD2 locus and to be flanked by

two single nucleotide polymorphisms (SNPs) at rs10972203 and rs6476455. Analysis of the sequence revealed a missense mutation in exon 2 of the σ-1R gene encoding a σ-1R (c.304G>C) which lead to the substitution of a glutamine for a glutamic acid at amino acid position 102 (E102Q), which was not found to be present in population-matched controls. This mutation is located in a predicted transmembrane domain, and has also been thought to be a part of a stretch of amino acids, which have been shown to be important for ligand binding. *In silico* analysis showed that the glutamic acid at position 102 of the σ-1R gene is highly conserved across vertebrates. The E102Q mutation was also shown to increase apoptosis in NSC-34 cells transfected with Sig-1R^{E102Q}-FLAG (Al-Saif *et al.*, 2011), which could prove to be important in neurodegeneration.

σ-1Rs have been shown to have chaperone activity at the ER. *In vivo* experiments have demonstrated that σ-1Rs are able to suppress the aggregation of misfolded proteins (Hayashi & Su, 2007), and this is very important in ER stress and the unfolded protein response. In addition, Ca^{2+} concentration in the ER is important for proper folding of nascent proteins. The loss of σ-1R function in neurons, such as motor neurons in the case of ALS, could be related to a loss of chaperone activity, which in turn increases misfolded proteins and leads to increased apoptosis. Increased apoptosis was shown in cells transfected with a mutated σ-1R variant that may cause stressed motor neurons to accumulate unfolded proteins and eventual degeneration (Al-Saif *et al.*, 2011).

σ-1R Involvement in Diseases

Major Depressive Disorder

Major depressive disorder is a common mood disorder characterized by depressed mood, insomnia, irritation, and cognitive deficits, and in severe cases can lead to suicide. It has been suggested that σ-1 receptor agonists can exert an effective antidepressant activity (Hayashi & Su, 2004). Following σ-1 receptor agonist administration, the potentiation of NMDARs or cholinergic neurotransmission been shown to improve cognitive activity in various amnesia models. In a study by Bergeron *et al.*, (1993), it was demonstrated that antidepressants such as sertraline and clorgyline, could behave as σ-1 receptor agonists by selectively potentiating the effect of NMDA, in a haloperidol-sensitive manner, on pyramidal CA3 dorsal hippocampus region of the rat. In animal models of depression it has been shown that σ-1R ligands, such as igmesine and SA4503, demonstrated antidepressant effects which were antagonized by the σ-1R antagonist BD1047 or NE-100 (Matsuno *et al.*, 1996).

Alzheimer's Disease (AD)

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disorder mainly manifested by cognitive and memory deterioration (reviewed by Blennow *et al.*, 2006; reviewed by Ishikawa and Hashimoto, 2009). There are several proposed models regarding the progression of pathogenesis in AD, but the most widely accepted is the amyloid cascade model**.** In brief, the model states that accumulation of oligomerized amyloid-β is an early event in AD patients, and leads to neurodegeneration (reviewed by Minati *et al.*, 2009). Postmortem analysis has revealed that the density of σ-1Rs is reduced in the hippocampus of patients with AD (Jansen *et al.*, 1993). As previously

mentioned, σ-1 receptor ligands have shown anti-amnesic or antidepressant effects and this was tested in AD relevant models of amnesia. Selective σ-1 receptor compounds like (+)-PTZ, PRE-084 or SA4503, in a dose-dependent manner attenuated the memory deficits observed in mice 7 days post β_{25-35} injection (Maurice *et al.,* 1998). In cultured cortical neurons, β_{25-35} peptide-induced neuronal death was shown to be blocked by the σ-1 receptor agonist PRE-084, an effect reversed by the σ-1 receptor antagonist NE-100 (Marrazzo *et al.,* 2005). Taken together, these studies have shed some light on the potential use of σ-1 receptors in the treatment of AD by their ability to alleviate cognitive deficits and reduce neuronal damage.

Schizophrenia

Schizophrenia is a major psychiatric disorder, which shows both positive (hallucinations and delusions) and negative (alogia, affective flattening, or avolition) symptoms as well as cognitive impairment. It has been shown that the traditional antipsychotic, haloperidol, binds to the σ-1 receptor with high affinity, although the prospective involvement if σ-1Rs in schizophrenia has not been clearly demonstrated. A few studies looking at σ-1R gene polymorphisms have been conducted, with conflicting results. One study demonstrated polymorphisms at two regions with a significant association with the TT/Pro2 schizophrenia haplotype (Ishiguro *et al.,* 1998). Another study identified another two polymorphisms of σ-1R genes in the 5'-upstream region, but could not find an association between these polymorphisms and schizophrenia (Satoh *et al.,* 2004). Due to the conflicting results regarding σ-1R gene polymorphisms and their association with the incidence of schizophrenia, the question of their potential relationship remains unanswered.

Addiction

Cocaine is the most well studied drug of abuse in relation to the σ-1R and one of the main reasons for this is that cocaine possesses a moderate affinity for σ-1Rs (Matsumoto *et al.*, 2003). Cocaine binding to σ-1Rs causes a dissociation of BiP from the σ-1R, which then allows σ-1R to translocate and interact with other proteins potentially involved in cocaine addiction. It has been shown that σ-1R antagonists significantly inhibited the convulsions and lethality induced by toxic doses of cocaine, and that σ-1R agonists potentiated the toxicity of cocaine (Matsumoto *et al.*, 2003). This finding gives clues into the potential involvement of σ-1Rs in the pathology of cocaine addiction, and its toxicity.

Ischemia

The neuroprotective role of σ-1Rs in models of ischemia has generated great interest in the potential therapeutic targeting of these receptors to enhance neuronal survival following stroke. The modulation on ion channels *via* σ-1Rs is not a new concept, as σ-1Rs have been shown to modify cell membrane excitability by regulating the activity of $K^{+,}$ Ca^{2+}, Na^{+}, and Cl^{-} ion channels. However, the mode of action of the σ-1R in the regulation of these ion channels remains unknown. The dysregulation of intracellular Ca^{2+} stores is largely responsible for the demise of neurons following an ischemic episode in the CNS (reviewed by Katnik *et al.*, 2006). The elevation of intracellular calcium disrupts plasma membrane function through the activation of intracellular calcium-sensitive ion channels and is responsible for the activation of downstream biochemical cascades involved in proteolysis, lipolysis, and the production of reactive oxygen species (reviewed by Katnik *et al.*, 2006). It could be speculated that since σ-1Rs have been shown to be involved in regulating the activity of Ca^{2+}channels, they could also be

involved in the dysregulation of intracellular calcium stores following an ischemic episode.

The involvement of σ-1Rs in neurological diseases has been of increasing interest because σ-1R agonists have been implicated in the enhancement of neuroplasticity, cognitive functioning, and neuroprotection. What is of explicit interest is how an ER resident protein is able to be involved in so many disease pathologies, and exactly how this unique receptor is distributed within the cellular environment, and hence how it exerts its actions. Many studies have shown the link between σ-1R and its downstream effect on target proteins, many of which include ion channels. However, the mode of action of the σ-1R in the regulation of these ion channels remains unknown.

σ-1Rs are able to modulate numerous ion channels and have been implicated in various neurological diseases. What is of great interest to our laboratory is the role of σ-1Rs in modulating *N*-methyl-D-aspartate receptors (NMDARs). NMDARs have been shown to be involved in a plethora of physiological aspects like learning and memory, and their dysfunction leads to numerous disease pathologies.

N-METHYL-D-ASPARTATE RECEPTORS (NMDARs)

Information transmission within the brain is dependent on complex variations in neuronal activity, and subtle variations in receptor function or distribution can drastically perturb the network. Electrical signals in the brain are thoroughly modulated and influenced by

both excitatory and inhibitory postsynaptic potentials (EPSP and IPSP, respectively), which are responsible for giving rise to an action potential (AP). An AP is a short-term change in the electrical potential in response to stimulation, resulting in the transmission of the electrical impulse. An AP travels from the somatodendritic compartment along an axon to its presynaptic terminal, where following a cascade of events, the release of neurotransmitters is triggered. Most excitatory synapses in the brain use the neurotransmitter glutamate to carry impulses between neurons. Glutamate has been shown to activate a mixture of NMDA and AMPA receptors in the postsynaptic cell. A staggering amount of research has been done looking at NMDARs and their involvement in excitatory synaptic transmission, and in turn their involvement in long-term potentiation, and learning and memory. What is fascinating about NMDARs is their multi-faceted properties which distinguish them from other ligand-gated ion channels.

NMDAR characteristics and Functional Domains

NMDARs are cationic channels that are permeable to sodium, potassium, and calcium. There are seven different NMDAR subunits, which falls into three subfamilies have been identified based on their sequence homology (reviewed by Traynelis *et al.*, 2010). A functional NMDAR is composed of two obligatory GluN1 subunits, and two GluN2 subunits, or a combination of GluN2 and GluN3 subunits. GluN1 and GluN2 containing NMDARs are the most abundant throughout the CNS, and are referred to as diheteromeric receptors as they are composed of two different subunits, and GluN2 subunits have been shown to determine channels properties and receptor localization. Triheteromeric receptors have also been shown to exist, and they are composed of two

GluN1, one GluN2, and one GluN3 subunit, and have been referred as "nonconventional" receptors (reviewed by Kehoe *et a*l., 2013).

All subunits share the same membrane topology characterized by three transmembrane domains, (TM1, TM3, and TM4) plus a re-entrant pore loop (M2) which lines the ion selectivity filter and form the channel pore, a large extracellular N-terminus region, an extracellular loop between TM3 and TM4, and an intracellular C-terminal domain, CTD (reviewed by Paoletti & Neyton, 2007). The CTD varies in length depending on the subunit and contains numerous sites of interaction with many intracellular target proteins (reviewed by Dingledine *et al.,* 1999). NMDARs also contains a highly conserved ligand binding domain (LBD) formed by two extracellular stretches of amino acids, S1 and S2, which adopt a clamshell-like conformation (reviewed by Dingledine *et al.*, 1999).

With the cloning of the GluN1 subunit in 1991 (Moyashi *et al*., 1991), GluN2 subunits in 1992 (Monyer *et al*., 1992, Kutsuwada *et al.,* 1992), and the GluN3 subunits in 1995 (Ciabarra *et al.,* 1995), the molecular bases for their structural and functional properties could be better defined. Although there is only one GluN1 subunit, through alternative splicing, eight possible splice variants can be generated. There are four distinct GluN2 subunits (GluN2A−D) encoded by four different genes, and there is also two GluN3 subunits (GluN3A−B) arising from two separate genes.

NMDAR Genes and Structure

GluN1

The human GluN1 subunit gene, *GRIN1*, is located on chromosome 9q34.3 with an approximate molecular mass of 130 kDa (reviewed by Dingledine *et al.,* 1999). The *GRIN1* gene has an open reading frame of 938 amino acids and a total of 22 exons, three of which (exon 5, 21, and 22) undergo alternative splicing to generate eight possible GluN1 splice variants (Zukin & Bennett, 1995). Four of these are generated through the alternative splicing of the C-terminus domain (Wenthold *et al.,* 2003). Exon 5 contains a 21 amino acid splice cassette, which is inserted into the N-terminal domain and contains six positively charged residues and three negatively charged residues (Zukin & Bennett, 1995). The inclusion of exon 5 controls proton and zinc inhibition among various kinetic properties (Vance *et al.,* 2012). Exon 21 and 22 encode two independent splice cassettes of 37 and 38 amino acids (C1 and C2, respectively) and make up the last stretch of the C-terminus domain (Zukin & Bennett, 1995). The various CTD splice variants are generated by the deletion of exon 21 or through alternative splicing of exon 22, or both, such that the C1 and/or C2 cassette are removed. The GluN1 CTD contains several important motifs shown to regulate receptor trafficking and binding to target proteins (reviewed by Cull-Candy & Leszkiewicsz, 2004).

GluN2 and GluN3

The GluN2 subunits are considerably larger (GluN2A-B have an approximate molecular mass of 170−180 kDa, while GluN2C-D have an approximate molecular mass of 150

kDa) and share only 20% homology with the GluN1 subunits. In the case of GluN2A and GluN2B, much of the additional mass resides in the extensive extracellular C-terminal domain (Zukin & Bennett, 1995). The GluN2 (GluN2A−D) subunits are encoded by four separate genes (GRIN2A−D, respectively), and are scattered on different chromosomes (reviewed by Dingledine *et al.,* 1999). The GluN3 subunits are the most recently discovered and were first identified in the mouse genome (Ciabarra *et al.,* 1995; Sucher *et al.,* 1995). The human GRIN3A gene shows close to 93% sequence identity to the rat GRIN3A, and is located on chromosome 9q34 in the region 13−34 and has a molecular mass of approximately 100 kDa (Anderson *et al.,* 2001).

As each of the NMDAR subunit has different properties, alternate compositions of subunits in a single functional NMDA receptor can have different functional properties. Through the use of subunit mRNA and protein distribution studies, and various other techniques, the channel properties associated with specific subunits have been studied.

Differences Between Subunits

Subunit composition plays a critical role in receptor function and activity. Apart from the difference in molecular mass, NMDAR subunits show several different functional properties. One of the main distinctions between the NMDAR subunits is their different deactivation times. Diheteromeric receptors composed of GluN1 and GluN2A subunits have very fast deactivation kinetics, in the millisecond range, while a GluN1 and GluN2D heterodimers have deactivation kinetics in the range of several seconds following a brief application of glutamate (reviewed by Cull-Candy *et al.,* 2001). Apart

from kinetic differences, another important difference between NMDAR subunits is their single-channel conductance and their block by extracellular Mg^{2+}. Diheteromeric GluN2A or GluN2B-containing NMDA receptors generate high conductance channel openings, with high Mg^{2+} block sensitivity (Dingledine *et al.,* 1999). On the other hand, GluN2C- and GluN2D-containing receptors generate low conductance channel openings, with a much lower Mg^{2+} block sensitivity. GluN2B-containing NMDARs have also been shown to desensitize more slowly and take longer to recover than GluN2A-containing NMDARs (Fuller *et al.,* 2006; Cull-Candy *et al.,* 2001). Recent experiments have shown that GluN3-containing NMDARs can give rise to low conductance channel openings when paired with GluN2A-containing NMDARs (reviewed by Cull-Candy *et al.,* 2001). These triheteromeric receptors show a fivefold reduction in the relative Ca^{2+} permeability when compared to GluN1/GluN2 heterodimers, which do not seem to show a drastic change in their Ca^{2+} permeability. These properties result in an increase in the duration of channel opening, and therefore affect receptor activation.

NMDAR activation

NMDARs are cationic channels, which are permeable to sodium, potassium, and calcium. The calcium influx through NMDARs is one of the critical factors mediating many of the NMDAR specific physiological and pathogenic conditions. There are three elements required for NMDAR activation: relief of the Mg^{2+} block, presynaptic release of glutamate, and presence of either glycine or D-serine. At resting membrane potential, the pore of the NMDAR channel is blocked by physiological levels of extracellular Mg^{2+}, resulting in the NMDARs unique role as coincidence detectors. NMDAR activation

requires post-synaptic depolarization in order to relieve the Mg^{2+} block that coincides with presynaptic release of glutamate that binds to the GluN2 subunit. The third element required is the presence of glycine or D-serine to occupy a binding site present on the GluN1 subunit (Sanz-Clemente *et al.,* 2012). Since the composition of a functional NMDAR is dependent on different subunits each with special distinguishing properties, it is important to address the question as to how these subunits are trafficked and assembled.

NMDAR Trafficking and Assembly

Trafficking

The addition of new or recycled membrane proteins to the plasma membrane is essential for maintaining existing synaptic structures, as well as for the generation of new membranes during development and synaptic plasticity. This brings us to the very important question regarding how proteins are trafficked to their target destinations, and more specifically, how NMDARs are trafficked to the plasma membrane.

As is the case with many receptors synthesized in the ER, GluN1 subunits contain an ER retention signal to prohibit their premature transport from the ER to their target destination (reviewed by Traynelis *et al.,* 2010). During receptor assembly, binding of the GluN2 subunit masks the GluN1 retention signal, and promotes forward trafficking of the GluN1/GluN2 complex through the secretory pathway, towards the cell surface (reviewed by Lau & Zukin, 2007). The association with synaptic scaffolding proteins like synapse-associated protein 102 (SAP-102) also promotes the forward trafficking of

receptors from the ER to the postsynaptic membrane (Sans *et al.*, 2003). SAP-102 is a member of the membrane-associated guanylate kinase (MAGUK) family of PDZ proteins, and is very wide distributed in the CNS. SAP-102 is the major MAGUK expressed in early development in early newly formed synapses. In this study, Sec8 was identified as a binding partner to SAP-102 (Sans *et al.*, 2003). Sec8 is one of the eight known components of the exocyst complex, which is involved in the docking of exocystic vesicles with a fusion site on the plasma membrane (reviewed by Cole *et al.*, 2005). It is suggested that PDZ protein-receptor interactions are essential for NMDAR delivery to the synapses and they occur early in the secretory pathway (Sans *et al.*, 2003). Once functional receptors are delivered to the cell surface, it is important that they be stabilized, and if necessary internalized.

Phosphorylation of NMDAR subunits has also been implicated in trafficking and synaptic plasticity, as Fyn-dependent phosphorylation of Tyr1472 on GluN2B subunits is required for proper localization of GluN2B-containing receptors at synapses in the hippocampus and amygdala (Nakazawa *et al.*, 2006). Stabilization of GluN2A receptors at the cell surface is largely dependent on tyrosine phosphorylation, as tyrosine dephosphorylation triggers GluN2A-containing receptor internalization (Vissel *et al.*, 2001).

NMDAR trafficking is affected in neuropsychiatric disorders. In the case of drug addiction, such as cocaine, acute cocaine administration has shown to lead to rapid insertion of primarily GluN2A-containing NMDARs at synapses in the ventral tegmental

area, shown to be involved in reward seeking behaviors (Borgland *et al.*, 2006). This alteration in synaptic composition of NMDARs is thought to be vital to the acquisition of cocaine sensitization, and may be involved in drug craving. NMDARs trafficking has also been shown to be involved in AD and schizophrenia as well. In AD, high affinity binding of β-amyloid to NMDARs leads to receptor internalization (reviewed by Lau & Zukin, 2007). In the case of schizophrenia, activation of neureglin 1, a growth factor genetically linked to schizophrenia in humans, promotes rapid plasma membrane NMDAR internalization (reviewed by Lau & Zukin, 2007).

Assembly

Three models of NMDAR assembly have been proposed, all of which are thoroughly reviewed (Traynelis *et al*., 2010). In brief, the first model suggests that GluN1 and GluN2 homodimers form initially, and subsequently assemble to form a tetrameric receptor. The second model is similar to the first, proposing the initial formation of a properly folded GluN1 homodimer which forms a stable complex, followed by the addition of two GluN2 monomers, leading to the formation of the NMDAR tetramer. The third model, which is most widely believed, suggests the initial formation of a GluN1-GluN2 heterodimer, followed by tetramerization to form a functional NMDAR. It is still unclear how the addition of a GluN3 subunit fits into the stoichiometry of a functional NMDAR. It has been proposed that neither GluN2 nor GluN3 subunits can form functional homodimers like the GluN1 subunits (Schuler *et al*., 2008). The assembly of GluN3A-containing NMDARs has been proposed as a two-step process: first a GluN1 subunit associates with either a GluN2 or a GluN3 subunit and forms a heterodimer, secondly two heterodimers form the final tetrameric receptor complex (reviewed by Kehoe *et al*., 2013). Regardless

of which model one is subscribed to, the importance of distribution of different NMDARs both within a single cell (synaptically and extrasynaptically), and throughout the CNS is evident. Since GluN1/GluN2 heterodimers are most abundant in the CNS, we will shift our focus mainly on their distribution, and both function and dysfunction leading to potential disease pathology.

Distribution

Subcellular

NMDARs are highly expressed at synaptic sites and to a lesser degree at extrasynaptic sites. The high degree of expression is believed to involve an interaction of the receptor with proteins that are part of the postsynaptic density (PSD) family of proteins. It is possible that the extrasynaptic population is simply representative of receptors that have been delivered to the plasma membrane and are awaiting incorporation into the synapse (reviewed by Wenthold *et al*., 2003). This idea is supported by recent studies showing NMDARs can rapidly move between synaptic and extrasynaptic sites, possibly by lateral diffusion of receptors (Tovar & Westbrook, 2002). There is also the theory that extrasynaptic receptors have a specific function, which involves their ability to respond differently to excitotoxic drugs (Sinor *et al*., 2000) and may be activated under physiological conditions (Chen *et al*., 2002).

System Wide

NMDARs are widely expressed and distributed throughout the CNS, although distribution of different receptor subtypes is highly dependent on brain area and developmental stage. The presence of GluN2A- or GluN2B-containing NMDARs differentially influences synaptic plasticity.

GluN1 subunits are ubiquitously expressed throughout the CNS in mature tissues. In situ hybridization studies looking at GluN1 mRNA throughout development found that in the fetal brain, GluN1 expression was low and specifically restricted to the cortex and hippocampus, and as development progressed, its expression increased and became more widespread (Monyer *et al*., 1992; summarized by Chaffey & Chazot 2008).

GluN2 subunits display distinct but similar expression patterns to those of GluN1 subunits throughout development. They are highly expressed throughout the hippocampus, cerebellum, thalamus and cerebral cortex. GluN2A is ubiquitously expressed in the CNS and its expression is evident at embryonic days 18-20, with expression increasing as development progresses. GluN2A expression is very low at the time of birth, and by post-natal day 22 GluN2A expression has been shown to reach adult levels (Takai *et al*., 2003; reviewed by Chaffey & Chazot, 2008). GluN2B is widely expressed during prenatal development, and, in the adult brain it is restricted to the forebrain (reviewed by Sanz-Clemente *et al*., 2012). GluN2C expression is first detected in the postnatal stages of development and it is highly enriched in the adult cerebellum, while GluN2D is present early in development and is strongest in the diencephalon,

mesencephalon, and spinal cord in adulthood (reviewed by Sanz-Clemente *et al*., 2012). The differential distribution of NMDARs both at the cellular level, and throughout the CNS is able to hint at specific functional properties of these subunits. There are many studies suggesting that GluN2A-containing NMDARs are involved in neuronal survival, (Stocca & Vicini, 1998; Tovar & Westbrook 1999), while extrasynaptic GluN2B-containing NMDARs have been associated with neuronal death and neurodegeneration (reviewed by Lujan *et al*., 2012).

Function

GluN2A in neuronal survival

GluN2A-containing NMDARs have been shown to preferentially localize to the synaptic zone, and to be linked to intracellular signaling cascades that promote cell survival during CNS injury (Stocca & Vicini, 1998; Tovar & Westbrook 1999). One theory explaining the different roles of NMDAR subunits is based on the notion that the important factor is the location of the receptors, either synaptic or extrasynaptic. It has been demonstrated that in hippocampal neuronal cultures, the synaptic activation of GluN2A containing NMDARs activates CREB, enhances BDNF gene expression and this in turn activates anti-apoptotic signaling pathways (Hardingham *et al*., 2002). An in *vivo* model of a focal ischemic stroke in rats has shown GluN2As to protect against apoptotic signaling, partially due to the activation of an Akt-signaling pathway (Liu *et al*., 2007). In a study looking GluN2A function it was shown that there was an increase in neuronal cell death following GluN2A pharmacological blockade (Cheng *et al*., 2008) and another study demonstrated an increase in pro-apoptotic signaling following GluN2A blockade

(DeRidder *et al*., 2006). There are several pathways that have been shown to be involved in the neuroprotective functions of GluN2A and how dysfunction in these pathways are involved in neurodegeneration (summarized by Lujan *et al*., 2012).

GluN2B in Neuronal Death and Neurodegeneration

While GluN2A subunits have been shown to be neuroprotective, the opposite seems to be the case for GluN2B subunits. As mentioned before GluN2B expression is high in the early postnatal days, and rapidly declines with development. In the developed brain, GluN2B has been shown to be localized primarily extrasynaptically. GluN2B is a major player in NMDAR-mediated excitotoxicity, and GluN2B overactivation during CNS injury, or dysfunction couples to pro-apoptotic pathways. This has been shown to occur through the suppression of CREB-, ERK-, and PINK1-dependent survival pathways (summarized by Lujan *et al*., 2012). Activation of these mainly extrasynaptic GluN2B receptors inhibits nuclear signaling to CREB, reduces BDNF activity, and plays a role in mitochondrial dysfunction, ultimately leading to the induction of pro-apoptotic pathways and cell death (summarized by Lujan *et al*., 2012). Since GluN2B receptors have been shown to be involved in neurodegeneration, it is interesting to think that one could make the link between these primarily extrasynaptic receptors and neurodegenerative disorders such as AD, Parkinson's, and ALS, although the precise GluN2B-signaling in neurodegeneration remains to be determined.

NMDAR Dysfunction/Diseases

NMDARs have been implicated in neuronal survival and maturation, neuronal migration, fine-tuning and stabilization of synaptic connections, and learning and memory. Under normal physiological conditions, tight regulation of NMDAR expression and function is required. Their essential involvement in the CNS, and their widespread distribution means that disruption of NMDAR physiology is often evident in neuronal pathologies. As mentioned previously, excessive glutamate-mediated Ca^{2+} entry into cells can lead to excitotoxicity and neuronal cell death, and this mechanism has been implicated in acute diseases such as ischemia and chronic neurodegenerative diseases like ALS.

It is becoming increasingly recognized that many neuropsychiatric disorders are linked to synaptic defects and NMDAR dysfunction that could be due to altered subunit expression and composition, trafficking, localization, and activity. NMDAR hyperactivity causes excessive Ca^{2+} influx through NMDARs which ultimately leads to excitotoxicity and neuronal death, but NMDAR hypofunction is also not a good thing.

Several lines of evidence indicate that hypofunction of NMDARs may be a key feature in major human cognitive disorders, most particularly schizophrenia. Non-selective NMDAR channel blockers (like PCP or ketamine) disrupt memory formation and cause a schizophrenia-like syndrome in humans, which includes psychotic behavior as well as cognitive impairments (Tsai & Coyle 2002). Several studies investigating the potential mechanisms of NMDAR involvement in schizophrenia have found abnormal glutamate receptor expression. Grimwood *et al*. (1999) discovered an upregulation of GluN2B-

containing NMDARs in the superior temporal cortex while Gao *et al*. (2000) found a significant increase in GluN2B mRNA in the CA2 hippocampal region of postmortem schizophrenic patients in comparison to controls. It has also been shown that inhibition of NMDAR activity during cortical development produces a cognitive deficit phenotype relevant to schizophrenia in adults (Stefani & Moghaddam, 2005).

Recently, NMDAR antagonism has been explored in order to combat the glutamatergic excitotoxicity and subsequent neuronal death seen in dementia. Memantine is a non-competitive, low affinity NMDAR open channel blocker which has been clinically approved for the treatment of AD (summarized by Chaffey & Chazot, 2008). The preferential binding of memantine to excessively activated NMDARs seems to reduce the adverse effects seen with other antagonists (summarized by Chaffey & Chazot, 2008).

NMDAR dysfunction has also been implicated in ALS, as glutamate-mediated excitotoxicity via AMPARs and NMDARs can lead to cell death via Ca^{2+} toxicity and this could further translate to the disruption of metabolic processes, the induction of proteolysis, stimulating free radical production and damaging mitochondrial membranes (summarized by Chaffey & Chazot, 2008). Motor neurons are especially susceptible to Ca^{2+} overload, as under physiological conditions they have naturally reduced levels of Ca^{2+}-binding proteins and GluA2-containing AMAPRs, which are permeable to Ca^{2+} (summarized by Chaffey & Chazot, 2008). An up to 50% reduction in GluN1 mRNA expression in the ventral horn in motor neuron disease patients, when compared to control tissue has also been observed (Samarasinghe *et al.,* 1996). Since there is

significant overlap between the involvement of σ-1Rs and NMDARs in numerous disorders, it is important to take into account their association with one another.

Plasticity

Brief trains of high-frequency stimulation to monosynaptic excitatory pathways in the hippocampus cause an abrupt and sustained increase in the efficacy of synaptic transmission (Bliss & Lomo, 1973). This effect is long-term potentiation (LTP) and has since been found in all excitatory pathways in the hippocampus and other brain regions, and is thought to be the process which underlies learning and memory (Bliss & Collingridge, 1993).

Specific NMDAR antagonists have minimal effects on basal synaptic transmission but completely block the generation of LTP, and similarly preventing the rise in postsynaptic Ca^{2+} with chelators blocks LTP, while raising the amount of postsynaptic Ca^{2+} can mimic LTP (reviewed by Malenka & Nicoll, 1999). In the CA1 region of the hippocampus, LTP is input-specific, which means that when generated at one set of synapses by repetitive activation, the increase in synaptic strength doesn't normally occur in other synapses on the same cell (reviewed by Malenka & Nicoll, 1999). It is well accepted that NMDARs play a central role in learning, memory and synaptic development. LTP of synaptic transmission in the hippocampus is the leading experimental model for the synaptic changes, which may be responsible for learning and memory. Although LTP is the most studied form of synaptic plasticity in the hippocampus, others are of increasing interest, including long-term depression (LTD).

While LTP is characterized by a long-lasting increase in synaptic strength, LTD is characterized by a long-lasting decrease in synaptic strength; both processes are thought to be involved in the storage of information in the CNS. It has been proposed that LTD, working in conjunction with LTP, underlies storage of memory (Bear, 1996). LTD is mediated by persistent changes and results in changes in the proportion of both pre- and postsynaptic receptors (reviewed by Collingridge *et al.,* 2010). LTD can be induced by prolonged periods of low frequency stimulation, by pairing baseline synaptic stimulation with neuronal depolarization (reviewed by Collingridge *et al.,* 2010). NMDAR-induced LTP is mainly a postsynaptic phenomenon, resulting in the removal of AMPARs from the synapse, and there is evidence suggesting NMDAR-induced LTD can involve a reduction in the probability of glutamate release (reviewed by Collingridge *et al.,* 2010). Induction of NMDAR LTD after low-frequency stimulation has also been observed in CA1 neurons. There is a clear relationship between LTD and novelty exploration. Low-frequency stimulation during exploration of a novel environment, containing novel objects resulted in LTD, LTD was not inducible through the exploration of a familiar environment (Kemp & Manahan-Vaughan, 2004). Although the mechanisms behind the structural changes following LTD are not well understood, there is evidence suggesting the involvement of LTD in the formation and storage of memories. It is important to better understand the underlying mechanisms of these processes in order to ameliorate our understanding of their functions within the CNS, and potentially their involvement in disease pathologies.

σ-1R Modulation of NMDARs

Due to the importance of NMDARs in LTP and their implication in several cognitive pathologies, and the evidence that σ-1R ligands have been shown to improve memory and learning in animal models of amnesia, the potential interaction between σ-1Rs and NMDARs has been under a lot of investigation. The initial study showing that σ-1R ligands are able to potentiate the NMDA-induced neuronal firing in CA3 hippocampal neurons, an effect blocked by σ-1R antagonist (Monnet et al 1990). In a study by Chen *et al*. (2006) it was shown that chronic administration of the σ-1R agonist, DHEA sulfate, facilitated the induction of LTP, which was blocked by a σ-1R antagonist (Chen *et al*., 2006).

Using whole-cell recording in CA1 pyramidal neurons in the rat hippocampus, Martina *et al.* (2007) demonstrated that PTZ potentiated the NMDAR responses and LTP by preventing small conductance Ca^{2+} -activated K^{+} current channels that are known to shunt NMDAR responses (Martina *et al*., 2007). This data suggests that σ-1Rs and their ligands may be able to regulate NMDARs and therefore LTP, by blocking SK channels. Bergeron *et al.* (1994) have also shown that low doses of selective σ-1R ligands are able to selectively potentiate the NMDA-induced activation of pyramidal neurons in the CA3 region of the rat dorsal hippocampus, and this effect was abolished following administration of the σ-1R antagonist, haloperidol (Bergeron *et al*., 1994, Bergeron *et al*., 1996)

There are several studies from various laboratories, through the use of electrophysiological techniques, showing that σ-1R ligands enhance the frequency of NMDAR-induced action potentials, the amplitude of NMDAR-mediated field EPSPs (fEPSPs) and NMDAR-mediated synaptic responses (Liang & Wang, 1998; Marquis *et al*., 1989; Martina *et al*., 2007; Monnet *et al*., 1990; Sabeti *et al*., 2007). In addition to the availability of electrophysiological data, behavioral tests have also shown similar results. Evidence exists that the *in vivo* administration of several different σ-1R ligands improves cognitive behaviour in the form of learning and memory, of animals experiencing NMDAR-antagonism induced amnesia (Maurice, 2001; Maurice *et al*., 1994a; Maurice & Privat 1997; Maurice *et al*., 1994b; Maurice *et al*., 1998). There are three important aspects in regards to the σ-1R. The first is that low doses of σ-1R agonists enhance NMDAR function (Bergeron *et al*., 1997; Maurice *et al*., 1994c), while high doses of agonists do not (Fletcher *et al*., 1995; Fletcher *et al*., 1993). Second, the σ-1R enhancement of NMDAR function is only seen when NMDARs are activated, and there is Ca^{2+} influx through the NMDARs (Martina *et al*., 2007, Maurice & Lockhart, 1997). Lastly, the facilitation of NMDAR-mediated responses and the amelioration in learning and memory following the administration of σ-1R ligands can typically be observed 5−30 min depending of the mode of administration (bath application of ligands to slices, or the *in vivo* administration through different routes). This effect is potentially sustained for as long at 2 h (Bergeron *et al*., 1995; Bergeron *et al*., 1997), and in some cases, to days (Sabeti *et al*., 2007; Maurice *et al*., 1994a; Maurice *et al*., 1994b). In a study done by Bergeron *et al.* it was reported that the increase in frequency of NMDA-induced action potentials at 15−30 min post intravenous *in vivo* administration of σ-1R agonists in rats

can be sustained for 90−120 min (Bergeron *et al*., 1995; Bergeron *et al*., 1997a; Bergeron *et al*., 1997b). Although there are several studies demonstrating that σ-1R agonists can enhance NMDAR function, the mechanism by which this occurs remains largely unknown.

OBJECTIVES

By employing several biochemical techniques, this study aimed to investigate whether there is a change in NMDAR protein expression levels in the rat hippocampus following the administration of several σ-1R agonists at different time points (30, 45, or 90 min). We also investigated whether the potential change was protein synthesis dependent, as well as looked at surface expression levels of NMDAR subunits following agonist treatment.

The biochemical characterization of intracellular and surface expression of NMDARs provides the opportunity to further understand the mechanisms involved in the σ-1R modulation of NMDAR function.

HYPOTHESIS

σ-1R activation increases the intracellular protein expression of NMDARs, as well as surface expression in the rat hippocampus.

MODEL OF STUDY

As σ-1Rs and NMDARs are highly expressed in the hippocampus and it is a well-defined structure that is easily isolated and studied, we chose to perform our experiments in the hippocampus. Several σ-1R ligands have been shown to modulate NMDAR activity and

Ca2+ influx in the rat forebrain, especially in the hippocampus, and for this reason we chose to study the rat hippocampus. Since biochemical approaches are very helpful in looking at protein expression levels, we chose to use various biochemical approaches in our experiments.

METHODS

Animals

Male Sprague-Dawley rats (6–8 weeks old) were purchased from Charles River, Montreal, QC, Canada. Animals were acclimatized and housed under standard conditions and had access to standard chow and water *ad libitum*. All procedures in this study were carried out in accordance with the guidelines provided by the Canadian Council on Animal Care, conforming to the National Institutes of Health guidelines and were approved by the University of Ottawa Animal Care Committee. All animal procedures were performed under isoflurane anesthesia.

Antibodies

The following antibodies and their dilutions were used in this study: mouse monoclonal anti-GluN1 (1:10,000; Synaptic Systems GmbH, Goettingen, Germany); mouse monoclonal anti-GluN2B and mouse monoclonal anti-GluN2A (both 1:750; LifeSpan Biosciences, WA, USA); rabbit monoclonal anti-GluA1 clone C3T (1:2500; Millipore, MA, USA); rabbit polyclonal anti-GluA2/3/4 (1:3500; Cell Signaling, MA, USA); and goat polyclonal anti-σ-1R (1:250; Santa Cruz Biotechnology, CA, USA).

Drugs

SKF, BD1047, and BD1063 were all purchased from Tocris, MN, USA; while PTZ was purchased from Sigma Aldrich, MO, USA. Anisomycin was purchased from Bioshop, ON, CA. Prior to use, SKF, BD1047, and BD1063 were all directly dissolved in a

phosphate buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). PTZ was initially dissolved in warm 0.1 N HCl, and was diluted in PBS immediately before use. Anisomycin was initially dissolved in DMSO, and then diluted in PBS before use to a final DMSO concentration of 20%.

Drug administration

Based on previously published reports, rats (6–8 weeks of age) were injected *i.p.* with 2 mg/kg SKF, PTZ, PRE (Steinfels *et al.,* 1988; Miller *et al.,* 1992; Beskid *et al.,* 1998), or PBS as a vehicle control. Similar drug pharmacokinetics were assumed for both SKF and PRE, and the same working concentration was used for both. Hippocampi were isolated and collected 30, 45, or 90 min following *i.p.* injection, as brain concentrations of PTZ are maximal 20–30 min post-injection and negligible after 120 min (Medzihradsky & Ahmad, 1971). Osmotic minipumps (Alzet, CA, USA), containing BD1047 or BD1063 (both 2 mg/kg/day), with continuous drug delivery of 1µl/h were subcutaneously implanted in rats for 2 days, to ensure a steady-state agonist concentration. The 2-day protocol was used to take advantage of σ-1R downregulation observed following prolonged administration of BD1047 (Zambon *et al.,* 1997). The protein synthesis inhibitor, anisomycin (30 mg/kg), was injected *i.p*. 1 h prior to SKF or PTZ treatment (Wanisch & Wotjak, 2008).

Isolation of crude synaptosomal fractions

Differential centrifugation was performed as previously described with minor modifications (Figure 1A; Hallett *et al.,* 2008), and all steps were performed at 4°C. In

brief, isolated hippocampi were homogenized in homogenization buffer (20 mM tris-HCl, 320 mM sucrose, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, and 1× EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland), pH 7.4) using a Dounce Tissue Homogenizer. The total homogenate (TH) was centrifuged at 800 × g for 10 min and the resulting pellet (P1) and supernatant (S1) were collected. To obtain the pellet (P2) and the supernatant (S2), the S1 was further centrifuged for 15 min at 9,200 × g. P2 was subjected to hyposmotic lysis by resuspension in 750 µl of homogenization buffer containing 35.6 mM sucrose instead of 320 mM. The lysate was then incubated on ice for 30 min with occasional mixing before centrifugation at 25,000 × g for 30 min. The resulting supernatant (LS1) and the pellet (LP1) were collected. The pellet was solubilized in 300 µl of solubilization buffer (20 mM tris-HCl, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 1% NP40, 0.1% SDS, 0.1% Na-deoxycholate, and 1× EDTA-free protease inhibitor cocktail, pH 7.4) for 30 min with end-over-end rotation and then subjected to a short spin for 5 min at 16,000 × g. The resulting solubilized crude synaptosomal fraction (LP1) was collected and diluted >10x for protein measurement and all the other isolated pellets were resuspended in homogenization buffer. Protein concentration was determined by Bradford or DC assay (Bio-Rad, CA, USA) before resolving on SDS-PAGE and Western blot (WB).

Surface Biotinylation

To perform surface biotinylation experiments, we followed the protocol published by Dennis *et al.* (2011) with some modifications. All steps were performed at 4°C unless otherwise stated. Hippocampal slices (300 µm) from drug and PBS treated rats were

incubated with 0.5 mg/ml EZ-sulfo-NHS-SS-biotin (Pierce) in PBS for 20 min on ice to biotinylate surface proteins. Excess biotin was quenched and removed by washing the slices 6× with cold supplemented tris-buffered saline (TBS⊕; 50 mM tris-HCl, 150 mM NaCl, 20 mM $MgCl_2$, 20 mM $CaCl_2$, pH 7.6) before homogenization in lysis buffer (150 NaCl; 20 mM HEPES; 2 mM EDTA; 0.1% SDS; 1% Triton X-100; pH 7.4), sonication for 2× 10 s at 20% efficiency on a sonicator (Fisher Scientific Model 120 Series Dismembrator, Fisher Scientific, NH, USA). After clearing the lysate with 10 min centrifugation at 14,000 × g, the biotinylated proteins were captured with neutravidin beads. The neutravidin beads were collected by centrifugation at 2,000 × g for 30 s and bound proteins were collected. The neutravidin beads were washed 10 × with 1 mL of TBS⊕ (TBS⊕; 50 mM Tris/HCl, 150 mM NaCl, 20 mM $MgCl_2$, 20 mM $CaCl_2$, pH 7.6) containing 0.05% SDS and bound proteins were recovered from the beads with 400 µl of elution buffer (50 mM Tris HCl, 2% SDS, 1 mM DTT, pH 6.8) by boiling for 10 min. Protein concentration was determined using DC assay (Bio-Rad) before SDS-PAGE and WB.

Western blotting and statistical analysis

For all WB samples, 10 µg of protein was resolved on 10% SDS-PAGE and transferred onto PVDF membranes and developed using Luminata Crescendo (Millipore). Bands were detected using film and sub-saturated bands were used for quantification of the pixel intensities using ImageJ (Schneider *et al*., 2012). WB experiments were repeated 5–8 times from at least 4 different animals and band intensities were normalized to β-tubulin or β-actin before comparison to vehicle unless otherwise stated. The statistical

significance was determined using a two-tailed, unpaired Student's t-test with a P value of < 0.05 considered statistically significant. The number of animals is represented by “n”.

RESULTS

σ-1R activation leads to an increase in GluN2-containing NMDARs in the rat hippocampus

An increase in NMDAR-mediated responses following σ-1R activation has been shown, but the method by which this occurs remains unknown. While there are many scenarios by which this effect is mediated, we chose to focus on two. The first of which is that σ-1R activation could lead to an increase in the number of NMDARs present at the cell surface, and second that σ-1R activation may be involved in the trafficking of NMDARs resulting in redistribution of NMDARs without causing a change in overall NMDAR numbers. Through using a differential centrifugation technique as previously described (Hallett *et al*., 2008), we looked at how *in vivo* σ-1R activation affects NMDAR expression levels in the LP1 fraction (Fig 2A).

There are several pharmacokinetic factors to take into account when administering a treatment *i.p*. (such as blood-brain-barrier permeability and first pass metabolism), we performed a time course experiment looking at three different time points following σ-1R agonist administration. Hippocampi were isolated 30, 45, and 90 min following 2 mg/kg administration of a σ-1R agonist and the LP1 fractions were collected. A representative WB showing GluN2A expression following 30 or 45 minute SKF (top) or PTZ (bottom) when compared to a vehicle control (PBS) is shown in figure 1B. An *i.p*. injection of SKF resulted in no change in the investigated NMDAR expression level following 30 or

45 minute agonist treatment (Fig 2B, GluN2A, 100±4.8%, P=0.98 at 30 min and 122±12% when compared to vehicle, P=0.46 at 45 min post injection, n=5 for both treatments). Following the administration of PTZ for 30 and 45 minutes, similar results were obtained (Fig 1B, GluN2A, 82.1±12%, P=0.37 at 30 min and 102±9% of vehicle, P=0.90 at 45 min post injection, n=5 for both treatments).

A robust upregulation of GluN2A and GluN2B subunit expression was observed following a 90 minute treatment with SKF, PTZ and PRE (Fig 3). Injection of SKF resulted in a 262±11% increase in GluN2A expression and a 260±23% increase in GluN2B relative to vehicle (Fig 3A, 3B; $P<0.05$, n=5). Thus, σ-1R activation results to a significant upregulation of GluN2 subunits 90 min after agonist administration, and as a result, all subsequent experiments were performed at this time point.

In order to confirm that this upregulation was in fact due to σ-1R activation, the experiment was repeated with two other σ-1R agonists, PRE and PTZ. A similar increase was observed following a 90 minute *i.p*. injection of 2 mg/kg PRE (Fig 3C; GluN2A, 146±8.9%; GluN2B, 134±9.7% of vehicle, $P<0.05$, n=6). As expected, *i.p*. administration of 2 mg/kg PTZ resulted in an increase of GluN2 subunits (Fig 3D; GluN2A, 146±8.9%; GluN2B, 134±9.7% of vehicle, $P<0.05$, n=6).

Through these experiments we were able to show that activation of σ-1Rs using three different classical agonists resulted in an upregulation of GluN2-containing NMDARs.

No change in AMPAR expression levels following σ-1R agonist administration

Previous studies have shown that σ-1R agonists have no significant effect on AMPAR-mediated currents (Fletcher et al 1995). This would implicate that σ-1R agonist activation has no effect on the protein expression of AMPARs. In order to verify this, we looked at AMPAR subunits GluA1 and GluA2/3/4 expression levels (Fig 4).

Our results revealed no significant changes in the expression levels of the AMPAR subunits studied when compared to a vehicle control, following 90 min *i.p.* injection of either SKF (Fig 4A, 4B; GluA1, 84.2±2.8%, P=0.32; GluA2/3/4, 97.6±11% of vehicle, P=0.91, n=6), PRE (Fig 4C; GluA1, 109±12%, P=0.71; GluA2/3/4, 86.5±19% of vehicle, P=0.35, n=6), or PTZ (Fig 4D; GluA1, 108±14%, P=0.80; GluA2/3/4, 95.7±16% of vehicle, P=0.91, n=6). β-tubulin was used as a loading control.

Taken together our results show that σ-1R activation leads to a significant increase in expression levels of GluN2-containing NMDARs, and that this effect is specific to GluN2-containing NMDARs and no change in the studied AMPAR subunits was observed.

Upregulation of GluN2-containing NMDARs by σ-1R agonist activation is blocked by σ-1R antagonists

In order to be able to verify that the observed upregulation of GluN2-containing NMDARs following σ-1R agonist administration was in fact due to the activation of σ-1Rs, we used classical σ-1R antagonists BD1047 and BD1063. We used *in vivo* chronic administration of BD1063 and acute and chronic administration of BD1047. For chronic administration, rats were implanted with osmotic pumps infused with the antagonist BD1063 (2 mg/kg) or BD1047 (2 mg/kg) for 2 days to ensure that σ-1Rs were blocked before the animals were challenged with either SKF or PTZ (Fig 5, Fig 6). For acute BD1047 treatments, 2 mg/kg were administered via an *i.p.* injection 1 h prior to challenging with SKF, PTZ, or PRE.

Chronic BD1063 treatment alone had no effect on GluN2 subunit expression levels (Fig 5A, 5B; GluN1, 119±14%, P=0.20; GluN2A, 101±6%, P=0.92; GluN2B, 109±10% of vehicle, P=0.56, n=3). As expected, pretreatment with BD1063 abolished the SKF-mediated increase in NMDAR subunit expression levels (Fig 5A, 5B: GluN1, 103±21%, P=0.86; GluN2A, 89±11%, P=0.67; GluN2B, 88±13% of vehicle, P=0.61, n=3).

To confirm the data we obtained *in vivo* with BD1063, we repeated the experiment with another σ-1R antagonist, BD1047 (Matsumoto et al., 1995). As acute *i.p.* administration of BD1047, results in effective σ-1R blockade (Nguyen et al., 2005), we compared two routes of σ-1R antagonist administration (acute vs. chronic) using 2 mg/kg of BD1047.

One experimental group received BD1047 for 2 days using osmotic minipumps (chronic), while the other group received a single *i.p.* injection of BD1047 (acute) 1 h before σ-1R agonist injection. Figure 6A shows representative blots of NMDAR expression after chronic and acute administration of BD1047 *in vivo*. Chronic antagonist treatment alone did not elicit a change in the protein levels under investigation (Fig 5=6A, 6B: GluN2A, 111 ± 4.2%, P = 0.25; GluN2B, 97.5 ± 4.0% of vehicle, P = 0.77; n = 5). Likewise, GluN2 subunit expression levels were not altered following a single, acute *i.p.* injection of BD1047 (Fig 6A, 6B: GluN2A 126 ± 11% of vehicle, P = 0.27, n = 3; GluN2B, 104±3.3% of vehicle, P=0.64, n=3).

Chronic administration of BD1047 prevented the robust increase in GluN2 subunits observed 90 min after SKF administration (Fig 6C, 6D: GluN2A, 128 ± 14%, P = 0.33, GluN2B, 118 ± 6.5% of vehicle, P = 0.23, n = 5). When the experiment was repeated using PTZ, the expression levels of GluN2 subunits were not significantly changed following chronic BD1047 administration (Fig 6C, 6D; GluN2A, 101±14%, P=0.76; GluN2B, 118 ± 16% of vehicle, P = 0.64, n =5). There was also no significant change in GluN2 subunit expression levels when SKF was injected 1 h after acute administration of BD1047 (Fig 6E, 6F: GluN2A 114 ± 7.8, P = 0.27, GluN2B, 104 ± 9.6% of vehicle, P = 0.69, n = 3). Similar results were obtained when PRE was administered 1 h after σ-1R antagonist treatment (Fig 6E, 6F: GluN2A 94 ± 4.8%, P = 0.60; GluN2B, 111 ± 8.9% of vehicle, P = 0.34, n = 3).

Thus, these experiments demonstrate that both chronic and acute administration of one of two σ-1R antagonists are able to block the upregulation of GluN2 subunits observed following injection of any one of the three σ-1R agonists under study. Taken together,

our pharmacological experiments show that activation of σ-1Rs is mediating an increase in GluN2 levels in the rat hippocampus

The σ-1R mediated increase in GluN2-containing NMDAR subunit expression is protein synthesis dependent

The next question that we needed to address was whether the observed upregulation is protein synthesis dependent and in order to do this, we used the protein synthesis inhibitor, anisomycin.

Recently it has been shown that σ-1R activation is able to regulate the protein expression of a number of membrane-bound ion channels (Ishima *et al.*, 2008, Nishimura *et al.*, 2008, Crottes *et al.*, 2011). This led us to the possibility that the increase in GluN2-containing NMDAR subunits following σ-1R agonist administration could be due to de novo protein synthesis. If this were the case, the inhibition of protein synthesis should abolish the upregulation of GluN2-containing NMDAR subunits observed 90 minutes post σ-1R agonist administration.

To test this hypothesis, the protein synthesis inhibitor anisomycin (Wanisch & Wotjak, 2008) was administered 1 h before challenging the animals with σ-1R agonists. In order to be able to validate the efficacy of anisomycin to block protein synthesis, we monitored cFOS levels. cFOS is an immediate early gene, and therefore is a commonly used marker

for protein sysnthesis (Fischer et al 2004). The expression of cFOS following anisomycin administration (30 mg/kg) alone was significantly reduced (Fig 7A, 7B; 73±1% of vehicle, $P<0.05$, n=4) when compared with vehicle, which indicates a decrease in de novo protein synthesis. In addition, anisomycin administration alone did not alter the expression levels of GluN2-containing NMDAR subunits (Fig 7A, 7B; GluN2A, 94±4.4%, P=0.26; GluN2B, 90±15% of vehicle, P=0.42, n=4).

Pretreatment with anisomycin was able to abolish the SKF-mediated increase in GluN2-containing NMDAR subunit expression (Fig 7C, 7D; GluN2A, 102±7%, P=0.86; GluN2B, 116±8% of vehicle, P=0.20, n=5). Similarly, the PTZ-mediated increase was also abolished following anisomycin pretreatment (Fig 7E, 7F; GluN2A, 118±8%, P=0.28; GluN2B, 115±16% of vehicle, P=0.61, n=6). These results demonstrate that the increase in GluN2-containing NMDARs following administration of σ-1R agonists is due to de novo protein synthesis.

σ-1R activation leads to an increase in surface levels of NMDARs

So far we have demonstrated that agonist activation of σ-1Rs leads to an increase in GluN2-containing NMDARs which is protein synthesis dependent. One obvious question following these results was to determine whether these newly synthesized subunits can be inserted into the plasma membrane, resulting in an increase in the surface expression of NMDARs. To address this, we performed a series of biotinylation experiments 90 min following an *i.p.* injection of SKF.

Figure 8A shows a representative control blot of β-actin intensity in total and surface fractions following vehicle or SKF injection. Our protocol enables us to detect changes in surface protein levels with no significant contamination from intracellular proteins, such as β-actin, as there is minimal β-actin signal in our surface protein fraction (Fig 8A). As a loading control, we also probed for glycine receptors (GlyRs), and we observed no change in the surface levels following SKF administration (Fig 8B). A significant increase in surface GluN2A (242±20% of vehicle, $P<0.05$, n=3) and GluN2B subunit expression (289±21% of vehicle, $P<0.05$, n=3) was detected following *i.p*. injection of SKF (Fig 8B, 8C). It was interesting to see that the surface level of GluN1 subunits was also increased following σ-1R activation (Fig 8B, 8C; 129±3.8% of vehicle, $P<0.05$, n=3).

Increase in surface NMDAR subunit expression is abolished treatment with σ-1R antagonist

In order to demonstrate that this effect was mediated through the activation of σ-1Rs, we repeated the biotinylation experiments in the presence of a σ-1R antagonist, BD1063 using osmotic pumps and employing the same method as described above. The administration of BD1063 had no effect on NMDAR subunit surface expression in and of itself (Fig 9A; GluN1, 93±11%, $P=0.86$; GluN2A, 98.5±3.47%, $P=0.33$; GluN2B, 98±2.7% of vehicle, $P=0.09$, n=4). The administration of BD1063 in the presence of SKF completely abolished the SKF-induced increase in NMDAR subunit surface expression

(Fig 9B; GluN1, 95±11.4%, P=0.64; GluN2A, 103±11.2%, P=0.55; GluN2B, 106±15.3% of vehicle, P=0.90, n=4). As expected, there was no change in the surface levels of AMPAR-subunit GluA1 with BD1063 alone (98±8.7% of vehicle, P=0.68, n=4) or following administration of SKF (106±11% of vehicle, P=0.72, n=4) as we can see in Figure 10A and 10B. Similar results were obtained when looking at the GluA2/3/4 AMPAR subunit in the presence of BD1063 alone (Fig 10A, 10B; 90.8±0.5% of vehicle, P=0.63, n=4), and in with SKF administration (Fig 10A, 10B; 77±5.8% of vehicle, P=0.45, n=4). This series of experiments shows that agonist activation of σ-1Rs leads to the NMDA-specific subunit insertion in the plasma membrane.

DISCUSSION

The data presented demonstrates that σ-1R activation leads to a *de novo* protein synthesis dependent increase in GluN2-containing NMDARs (Fig 2−7). Following σ-1R activation, there is also an increase in surface expression of GluN2-containing NMDARs (Fig 8), while there is no change in the surface expression of NMDAR subunits following pretreatment with a σ-1R antagonist, BD1063 (Fig 9), with no change in AMPAR expression levels (Fig 10). These results provide some understanding into the σ-1R mediated specific modulation of NMDAR upregulation and surface expression in hippocampal neurons.

One interesting result is that σ-1R agonist activation did not alter GluN1 subunit expression levels (Fig 3). One possible explanation for this is that GluN1 subunit expression is regulated by a different mechanism than GluN2 subunits. Another is that the availability of GluN2 subunits may be the limiting factor in the formation of stable NMDARs. Previous work in cultured cerebellar neurons shows that the majority of GluN1 subunits reside in an intracellular pool with rapid turnover, and that this pool of GluN1 subunits is not associated with GluN2 subunits (Huh & Wenthold, 1999). Once the GluN1 subunits are associated with the GluN2 subunits, the resulting complex is efficiently trafficked to the plasma membrane (McIlhinney *et al.*, 1996; Huh & Wenthold 1999). Since σ-1Rs have chaperone functions, it is possible that they may be involved in the trafficking of NMDARs to the cell surface. There are several studies suggesting that σ-1Rs could form a macromolecular complex with NMDARs and that σ-1R agonists are able to modulate this interaction. Direct interactions between σ-1R and NMDARs have

been observed in recombinant systems, but there is a discrepancy with regards to the locus of this interaction. Balasuriya *et al*. (2013) demonstrate that the σ-1R NMDAR interaction is at the GluN1 subunit, while our laboratory demonstrate this interaction to be at the GluN2 subunits (Pabba *et al.,* 2014). Results from our laboratory suggest that the locus of interaction is between σ-1R and the GluN2A subunit. One problem with co-immunoprecipitation is that this technique does not tell us whether the interaction between σ-1R and GluN2A is direct or if it is a part of a larger complex.

Using label transfer protein interaction analysis, we will be able to study the potential interaction between σ-1R and NMDARs, by labeling proteins that interact with a protein of interest, through using a bait protein with a target protein, and transfected in cultures. The labeled bait protein is then allowed to interact with target protein to form a complex, after which the complex is exposed to UV light which in turn causes the formation of a covalent bond between the bait protein and the target protein. Another way to look at the interaction between σ-1R and NMDARs is to transfect fluorescently labeled, truncated forms of σ-1R as well as different NMDAR subunits, and activating the σ-1R with selective ligands. These will be transfected into the MCF-7 cell line, as it has been shown to not express σ-1R (reviewed by Wu & Bowen, 2008). Since a splice variant lacking exon 3 of the σ-1R does not have the ability to bind σ ligands, and two amino acids have been identified to be important in ligand binding (reviewed by Aydar *et al.,* 2004), we would need to work around these amino acids in order to be able to activate the σ-1R and its interaction with NMDAR subunits.

Through our biotinylation experiments we were able to show an increase in cell surface NMDARs following σ-1R activation (Fig 7). This may have an important functional role since NMDARs have been implicated in an array of physiological and pathological functions. As mentioned before, NMDARs are composed of two GluN1 subunits, and two GluN2 subunits. NMDARs in hippocampal and cortical neurons are often thought of as diheteromeric, which means that they contain only one type of GluN2 subunit. Triheteromeric receptors of the GluN1/GluN2A/GluN2B subtype have been observed in native tissues, most particularly in the cortex (Sheng *et al*., 1994), and GluN1/GluN2A/GluN2C receptors have been observed in the cerebellum (Chazot *et al*., 1999). Our biotinylation results suggest that σ-1R activation leads to the increased surface expression of GluN1, GluN2A, and GluN2B subunits. What our experiments can't tell us is whether these receptors are GluN1/GluN2A or GluN1/GluN2A/GluN2B. Another caveat to our experiments is that since we are doing surface biotinylation on hippocampal slices, we are potentially looking at other cell populations. Studies suggest that astrocytes express functional GluN2B-containing NMDARs after ischemia *in vivo* and anoxia *in vitro* (Krebs *et al*., 2003). It has also been shown that glia cells express functional NMDARs as well in the rat dorsal root ganglia (Castillo *et al*., 2013). More studies need to be conducted to conclusively determine whether NMDARs are expressed in other neuronal cell populations.

The localization of GluN2B-containing receptors both synaptically and extrasynaptically provides the potential for increased NMDAR activity through the release of glutamate within and externally of the synapse. Recent findings suggest that extrasynaptic

NMDARs promote neuronal plasticity through a contribution to the calcium signaling necessary to induce these changes (Harris & Pettit, 2008). Further, it has been shown that specific frequency stimulation trains are able to activate all synaptic and extrasynaptic dendritic NMDARs, suggesting that these NMDARs act as synaptic receptors as needed to transiently increase synaptic strength (Harris & Pettit, 2008). These extrasynaptic NMDARs thus play an important role in synaptic physiology, and call into question their status as "extrasynaptic". The GluN2B subunit has also been associated with vital behavioural and physiological functions like feeding, learning, and memory (Loftis & Janowsky, 2003). Although it has been suggested that extracellular activation of GluN2B receptors is primarily associated with pro-apoptotic pathways, more studies need to be conducted to verify the validity of these results, as there are others suggesting the importance of extracellular GluN2B receptors in normal physiological function of neurons. Our data shows that surface GluN2A receptor expression is increased following σ-1R activation and GluN2A receptor activation has been shown to preferentially be associated with promoting cell survival through the activation of various anti-apoptotic cell mechanisms, but this needs to be further studied.

In order to show whether the increase of cell surface GluN2A and GluN2B subunits is observed in neurons and whether the localization of these receptors is synaptic or extrasynaptic, is to do 2-Photon (2P) laser imaging and whole-cell recordings in slices. We will fill CA1 neurons *in situ* with the fluorescent dye Alex 594, which would allow us to morphologically visualize specific dendrites and spines. After which MNI-glutamate will be released with a brief IR laser illumination. This causes the caged

glutamate to be uncaged, and therefore activated, causing the activation of NMDARs at a very specific spot. We will monitor the effect of σ-1R activation on the modulation of NMDARs by bath applying SKF on neurons, and measure 2P-EPSCs mediated by NMDARs. We expect that the uncaging of glutamate on dendritic spines in the presence of SKF will give very large EPSCs when compared to control recordings, since according to our biotinylation data both GluN2A- and GluN2B-containing NMDARs are enriched following σ-1R activation. Since we will be morphologically identifying and selecting neurons, this experiment will give us insight on whether the observed increase in GluN2A and GluN2B in our biotinylation experiments is in fact coming from neurons or other cell populations. If we see an increase in GluN2A EPSCs following σ-1R activation in our 2P study, but not in GluN2B EPSCs, it is indicative that the increase in GluN2A expression in our biotinylation studies is coming from neuronal cells, but not the GluN2B enhancement – which would mean that the GluN2B increase in expression is due to an increase in other cellular populations (*e.g.* glial cells or astrocytes). The reverse would be true if we see an increase in GluN2B EPSCs but not in GluN2A EPSCs. Since GluN2A-containing NMDARs are thought to be primarily localized synaptically, and GluN2B-containing NMDARs extrasynaptically, the results of this experiment will shed some light on the localization of the newly inserted cell surface NMDARs following σ-1R activation.

Through the series of these proposed experiments, we have been able to address the questions of where the locus of interaction between σ-1R and GluN2A is, and whether the observed increase in cell surface expression of Glun2A and GluN2B following σ-1R

activation is neuronal or non-neuronal in nature, and whether it is synaptic or extrasynaptic. The next question to address is whether this increase in expression is due to an increase in both GluN2A and GluN2B diheteromeric receptors, or an increase in GluN1/GluN2A/GluN2B triheteromeric receptors.

A way to address this issue is to selectively express triheteromeric receptors and compare their properties with diheteromeric receptors, as was done by Hansen *et al.* (2014). Through this technique, we will generate three distinct populations of transfected HEK-293 cells: the first two will express only either GluN2A or GluN2B diheteromeric receptors, and the last will express only triheteromeric receptors. We will look at evoked currents following σ-1R agonist activation. If σ-1R preferentially modulates either GluN2A- or GluN2B-containing heterodimers, we expect to see a large increase in the evoked current in those cells following application of a σ-1R agonist, and not a significant increase in the evoked currents of the triheteromeric HEK cells.

What is exciting about our results is the novel idea that σ-1R activation enhances the cell surface expression of NMDARs. This is very important since NMDARs have been implicated in several neurodegenerative diseases, such as AD, ALS, Parkinson's and many more, as well as their involvement in learning and memory. Progressive, age-related deficiency in learning and memory is a staple in AD, as well as the pathological hallmark including neurofibrillary tangles associated with β-amyloid and permanent neuronal cell loss. NMDA receptor composition has been shown to differ between unaffected individuals and Alzheimer's cases. In contrast to the GluN2A isoform, GluN1

and GluN2B levels are decreased in human postmortem brains in Alzheimer's disease (summarized by Villmann & Becker, 2007). Since we have been able to show that σ-1R activation leads to an increased expression of cell surface NMDARs, one could speculate that this effect would be desired in the development of potential Alzheimer's therapeutics. Due to its hypothesized neuroprotective nature, one could speculate that increased expression of GluN2A in disease models such as ALS could be beneficial. More studies need to be done to further dig into functional and physiological implications of this.

Although many more studies need to be done looking at the role of σ-1R regulation of NMDARs and their potential role as therapeutic targets in numerous neurodegenerative disorders, our data suggests that the link between the two is there. We have shown that there is a *de novo* protein synthesis dependent increase in GluN2 subunits following σ-1R activation, and these newly synthesized receptors are inserted into the plasma membrane.

CONCLUSION

There is a plethora of evidence available demonstrating the functional interaction between σ-1Rs and numerous ion channels, one specific to our interest being the NMDA receptor. Although the functional evidence exists, mechanistic evidence has been more elusive to gather. It is currently unknown the exact mechanism as to how σ-1Rs enhance NMDAR functions. The results presented in our study give some insight regarding this mechanism. As both σ-1Rs and NMDARs have been implicated in numerous disease pathologies, it is important to fully understand their interaction and involvement in both normal conditions, and disease states. This σ-1R – NMDAR partnership could prove to be a useful tool in developing novel therapeutic strategies to treat a multitude of diseases, including AD, schizophrenia, depression, addiction, ALS, and stroke.

TABLES

Compound	Subtype Selectivity	σ-1 site K_i or K_D	Function on σ-1 site
Benzomorphans			
(+)-Pentazocine	σ-1	+++	Agonist
(–)Pentazocine	σ-1/ σ-2	++	Agonist
(+)-SKF10,047	σ-1	+++	Agonist
Antipsychotics			
Chlorpromazine	σ-1/ σ-2	++	?
Haloperidol	σ-1/ σ-2	+++	Antagonist
Nemonapride	σ-1/ σ-2?	+++	?
Antidepressants			
Clorgyline	σ-1	+++	Agonist?
Fluoxetine	σ-1	+	Agonist
Fluvoxamine	σ-1	+++	Agonist
Imipramine	σ-1	++	Agonist
Sertaline	σ-1	++	Agonist
Antitussives			
Dextromethorphan	σ-1	++	Agonist
Dimemorfan	σ-1/ σ-2	++	Agonist
Parkinson's and/or Alzheimer's disease			
Donepezil	σ-1/ σ-2?	+++?	Agonist
Memantine	?	+	Agonist
Drugs of Abuse			
Cocaine	σ-1/ σ-2	+	Agonist
Metamphetamine	σ-1/ σ-2	+	?
Putative endogenous ligands			
DHEAS	σ-1	+	Agonist
Pregnenolone sulfate	σ-1	+	Agonist
Progesterone	σ-1	+	Antagonist
Other Drugs			
BD1047	σ-1	+++	Antagonist
BD1063	σ-1	+++	Antagonist
DTG	σ-1/ σ-2	+++	?
NE-100	σ-1	+++	Antagonist
PRE-084	σ-1	+++	Agonist
SA4503	σ-1	+++	Agonist

Table 1. Pharmacology of common σ receptor ligands. The most common σ-R ligands with their specificity for the σ-1R compared to the σ-2R, level of affinity and function for the σ-1R. The affinities: + refers to < 10 μM, ++ refers to < 500 nM, and +++ refers to < 50 nM. The symbol "?" refers to information that has not yet been studied, or remains unclear at the moment. This table is modified from Cobos *et al.* 2008.

FIGURES

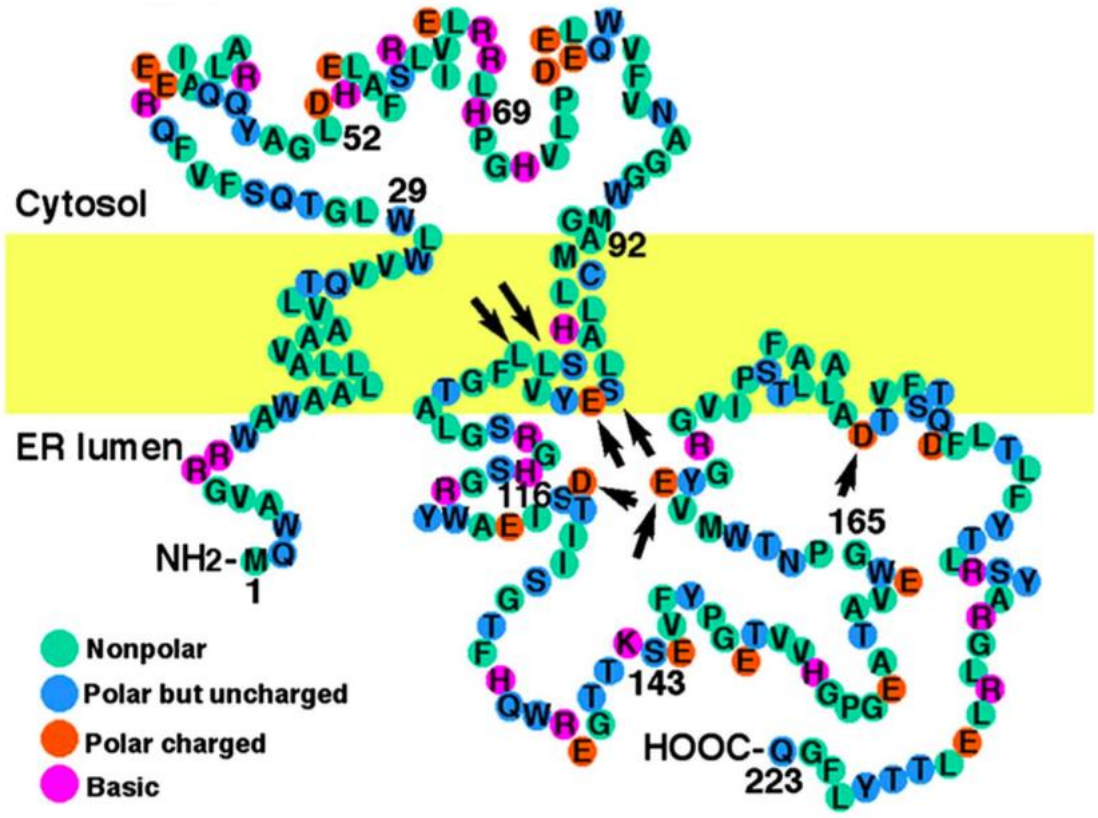

Figure 1: The proposed structure of the σ-1R.

The σ-1R consists of 223 amino acids, two transmembrane domains and three hydrophobic regions. The N- and C- termini are both localized to the ER lumen. The arrows represent potential ligand-binding sites, which are contained within the C-terminus. This image was acquired from a publication by Hayashi & Su, 2007.

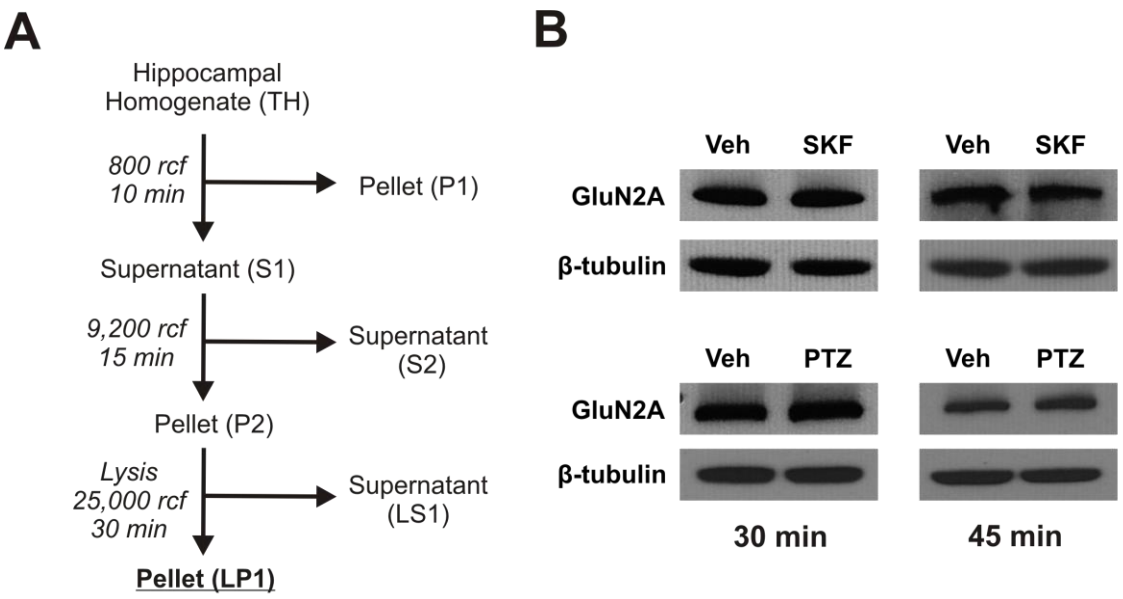

Figure 2: There is no increase in GluN2A subunit expression in the LP1 fraction 30, or 45 min post σ-1R agonist administration.

Schematic diagram showing the methodology used to isolate subcellular fractions from hippocampal homogenate (A). Representative WBs of LP1 probed for GluN2A expression 30 and 45 min after vehicle (Veh), SKF, or PTZ injection. β-tubulin was used as a loading control (B). This figure was modified from Pabba *et al*. (2014).

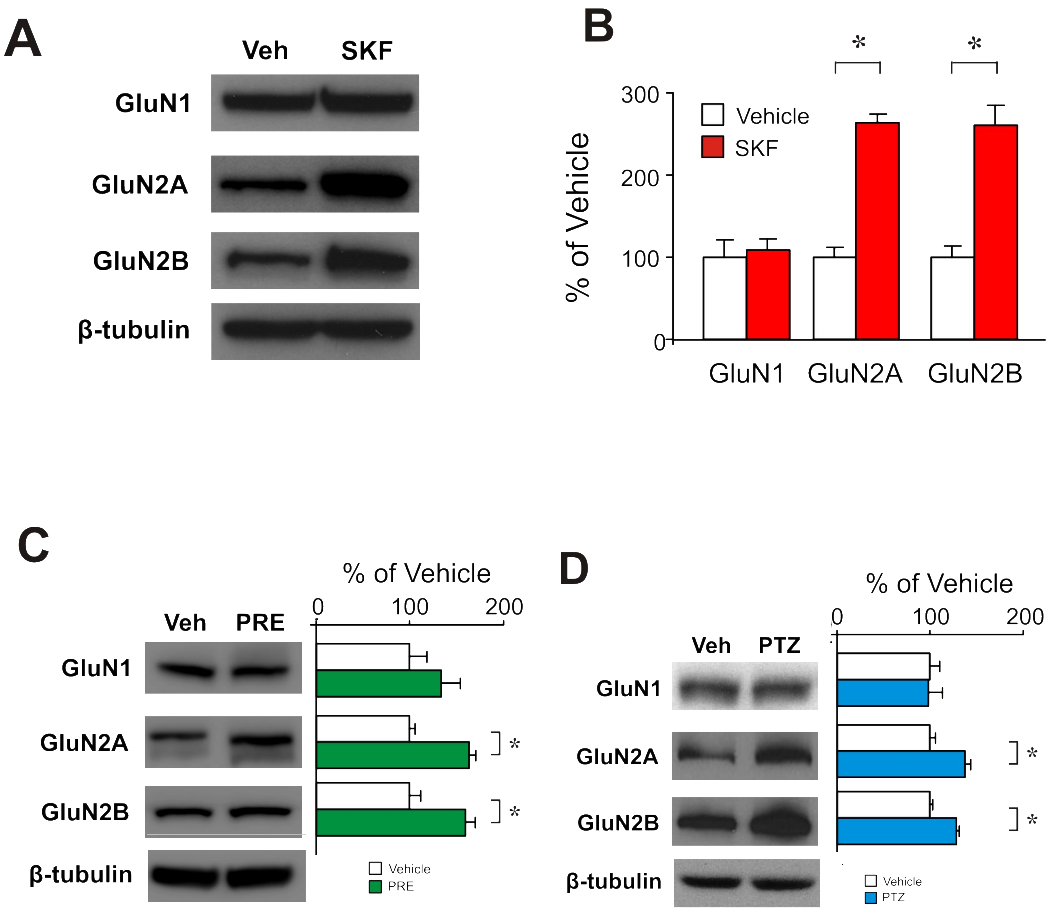

Figure 3: An increase in NMDAR subunit expression is observed following administration σ-1R agonists.

A significant increase in protein expression levels of GluN2A, and GluN2B was observed 90 min after an *i.p.* injection SKF when compared to vehicle (Veh) (A–B). Similar results were obtained 90 min after injection of PRE (C), or PTZ (D). β-tubulin was used as a loading control. Bar graphs are mean ±SEM of at least 5 animals. Asterisks indicate a statistical significance ($P<0.05$). This figure was modified from Pabba *et al*. (2014).

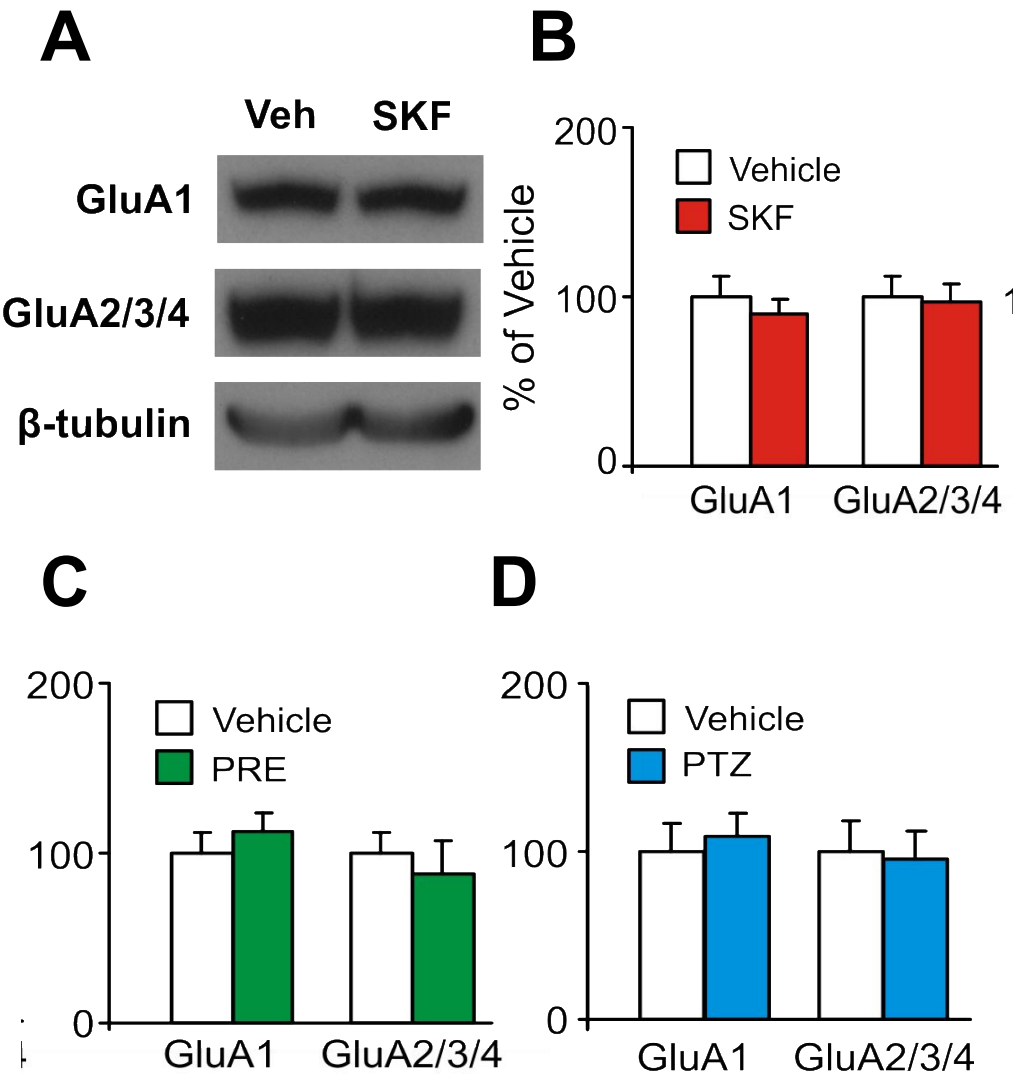

Figure 4: There is no change in AMPAR expression following the administration of σ-1R agonists.

A representative WB showing that there is no significant change in the expression levels of AMPAR subunits GluA1 and GluA2/3/4 90 min following the administration of SKF (A-B), PRE (C), or PTZ (D) when compared to a vehicle control (Veh). β-tubulin was used as a loading control. Bar graphs are mean ±SEM of at least 5 animals. This figure was modified from Pabba *et al*. (2014).

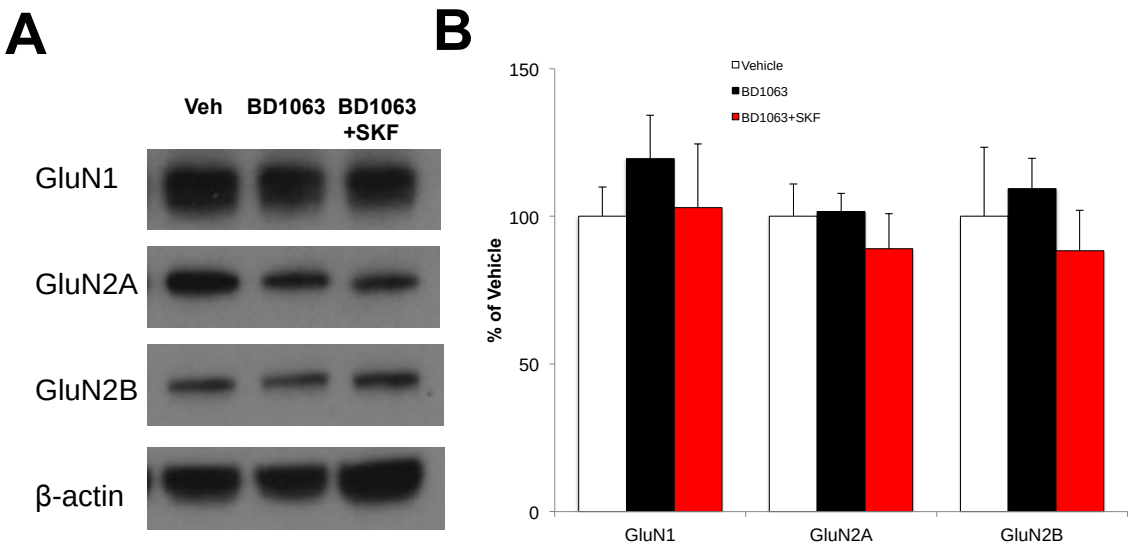

Figure 5: Chronic administration of BD1063 abolishes the σ-1R mediated increase in NMDAR subunit expression *in vivo*.

A representative WB showing that there is no significant change in the expression levels of NMDARs following a 2-day chronic administration of σ-1R antagonist BD1063 when compared to vehicle (Veh) (A). Chronic administration of BD1063 blocked the increase of GluN2 containing NMDARs observed 90 min following an *i.p.* injection of SKF (B). β-actin was used as a loading control. Bar graphs are mean ± SEM of at least 5 animals.

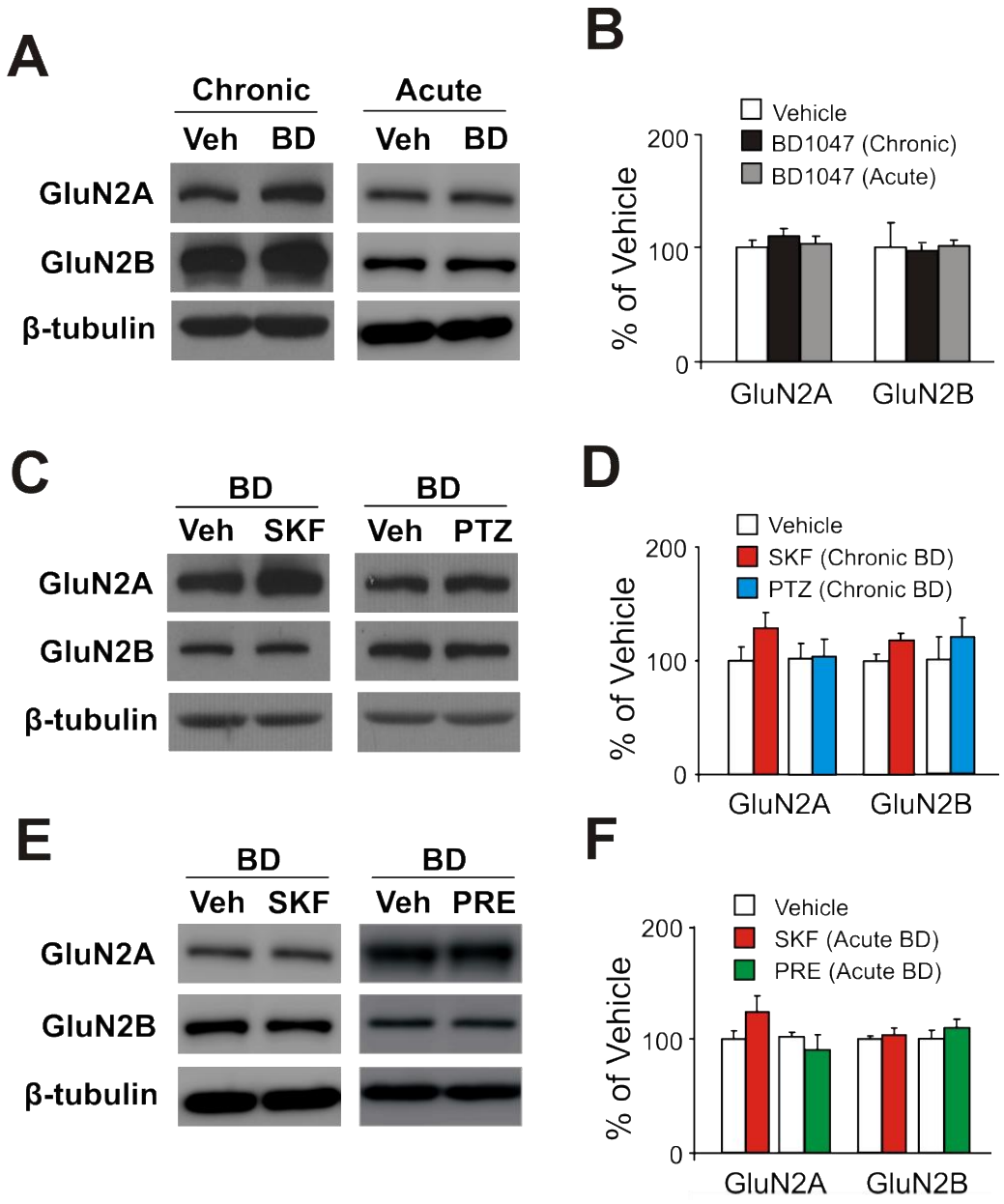

Figure 6: Chronic and acute administration of BD1047 abolishes the σ-1R mediated increase in NMDAR subunit expression *in vivo*.

There was no change in the expression levels of GluN2A or GluN2B following a 2-day chronic or acute administration (single *i.p.* injection) of the σ-1R antagonist BD1047 alone when compared to vehicle (Veh) (A–B). Chronic administration of BD1047 blocked the increase of GluN2 containing subunits observed 90 min following an *i.p.* injection of SKF or PTZ (C–D). Acute administration of BD1047 also abolished the increase in GluN2 containing subunits following SKF or PRE administration (E–F). β-tubulin was used as a loading control. Bar graphs are mean ± SEM of at least 5 animals. This figure was modified from Pabba *et al*. (2014).

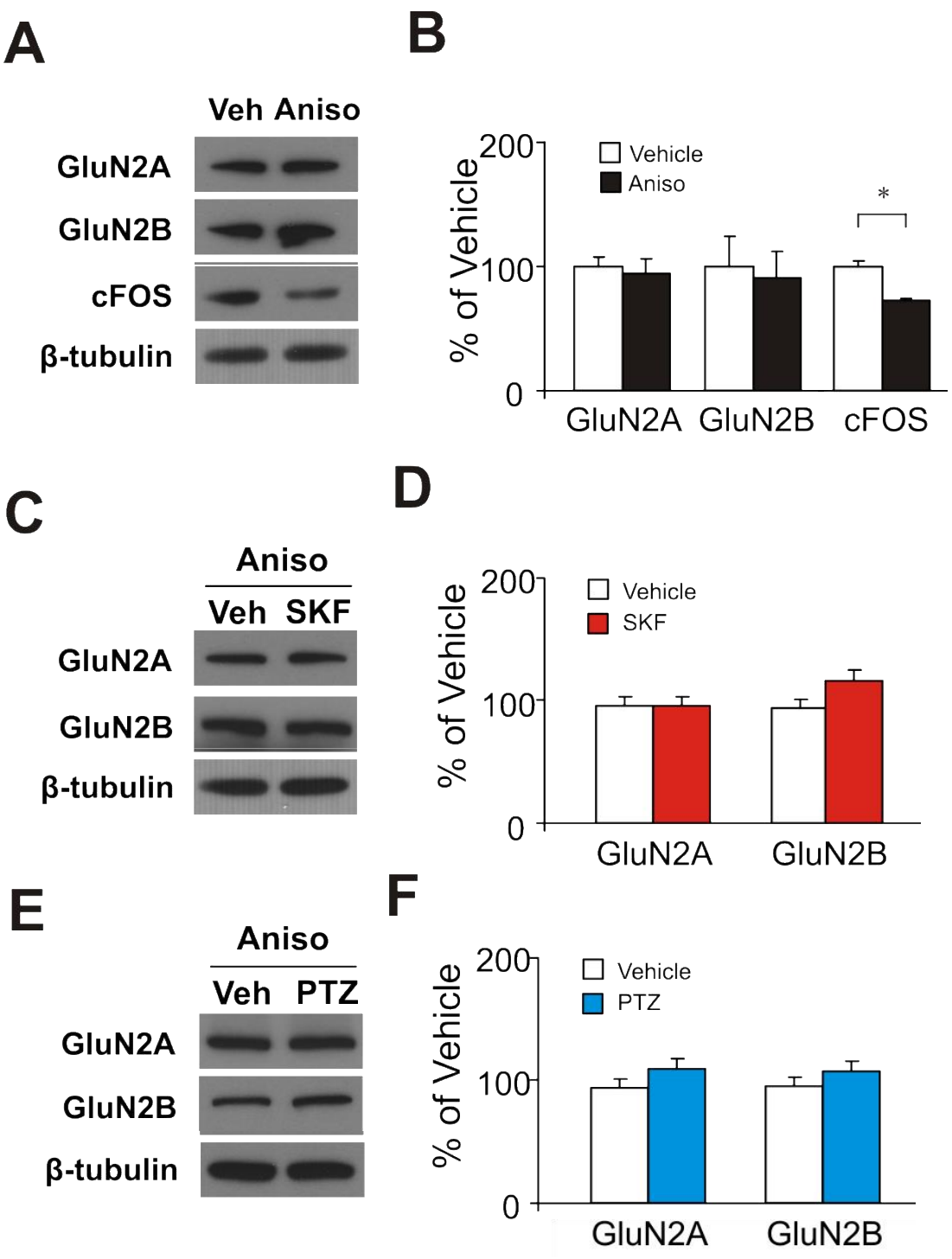
A
Veh Aniso
GluN2A
GluN2B
cFOS
β-tubulin
B
200
100
0
% of Vehicle
Vehicle
Aniso
*
GluN2A GluN2B cFOS
C
Aniso
Veh SKF
GluN2A
GluN2B
β-tubulin
D
200
100
0
% of Vehicle
Vehicle
SKF
GluN2A GluN2B
E
Aniso
Veh PTZ
GluN2A
GluN2B
β-tubulin
F
200
100
0
% of Vehicle
Vehicle
PTZ
GluN2A GluN2B

Figure 7: The increase in GluN2 subunit expression following σ-1R activation is protein synthesis dependent.

A representative WB showing the expression levels of GluN2A, and GluN2B were unaffected by *i.p.* injection of vehicle (Veh) or anisomycin (Aniso). An observed decrease in the levels of cFOS (B), demonstrating that anisomycin effectively blocked protein synthesis at this time point. Interestigly, anisomycin treatment prior to SKF (C–D) or PTZ (E–F) prior to administration prevented any change in the expression levels of GluN2 subunits. β-tubulin was used as a loading control. Bar graphs are mean ±SEM of at least 5 animals. Asterisks indicate a statistical significance ($P<0.05$). This figure was modified from Pabba *et al*. (2014).

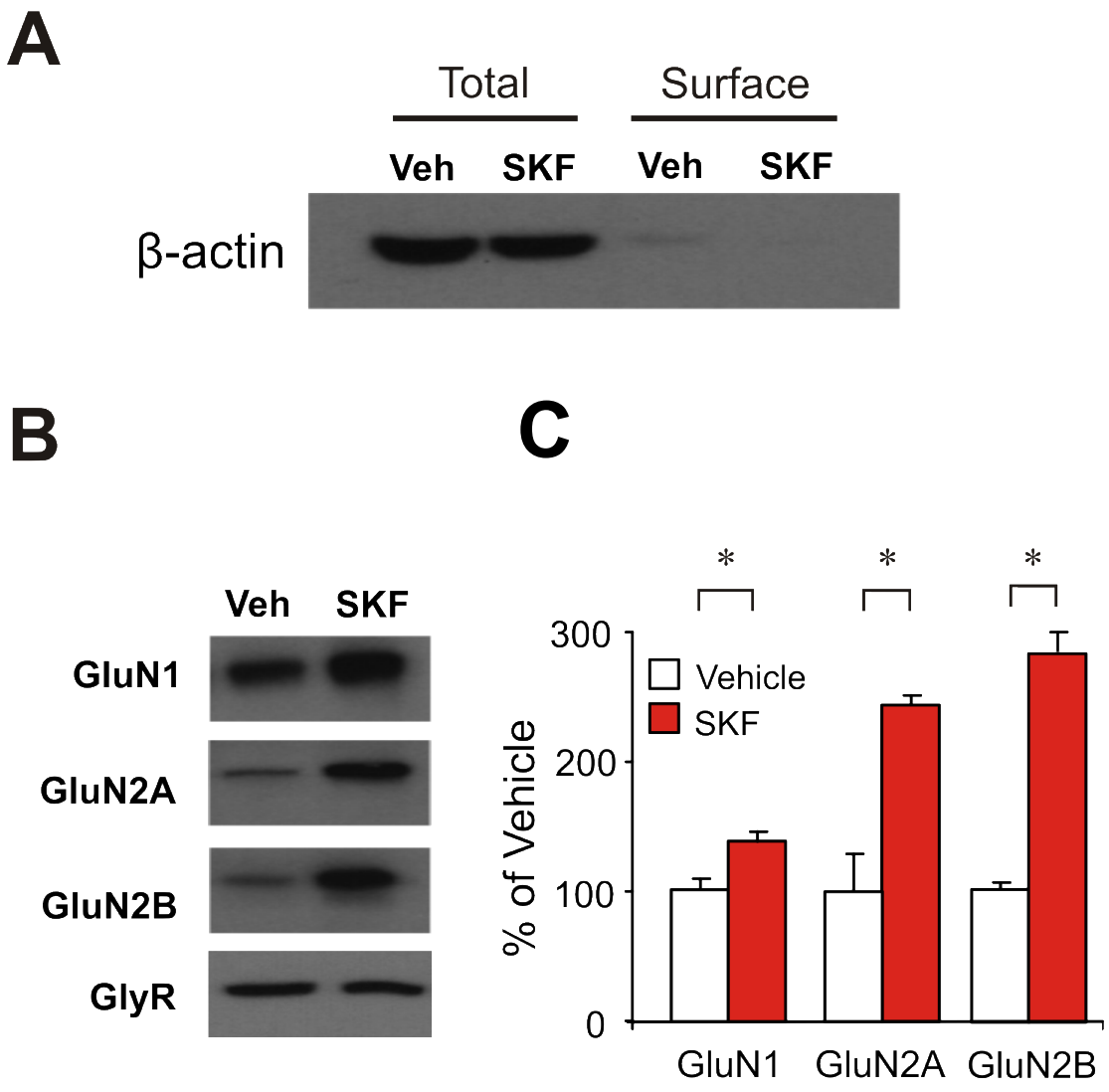

Figure 8: An increase in the surface levels of NMDARs was observed following SKF administration.

A representative WB of β-actin in the whole cell homogenate (Total) and the biotinylated (Surface) fractions showing a marked reduction in band intensity in the surface fraction, typical of an inctracellular protein (A). There was an upregulation in the surface levels of NMDAR subunits following *i.p.* injection of SKF compared to vehicle (Veh) (B–C). The Glycine Receptor (GlyR) was used as a loading control. β-tubulin was used as a loading control. Bar graphs are mean ±SEM of at least 5 animals. Asterisks indicate a statistical significance ($P<0.05$). This figure was modified from Pabba *et al*. (2014).

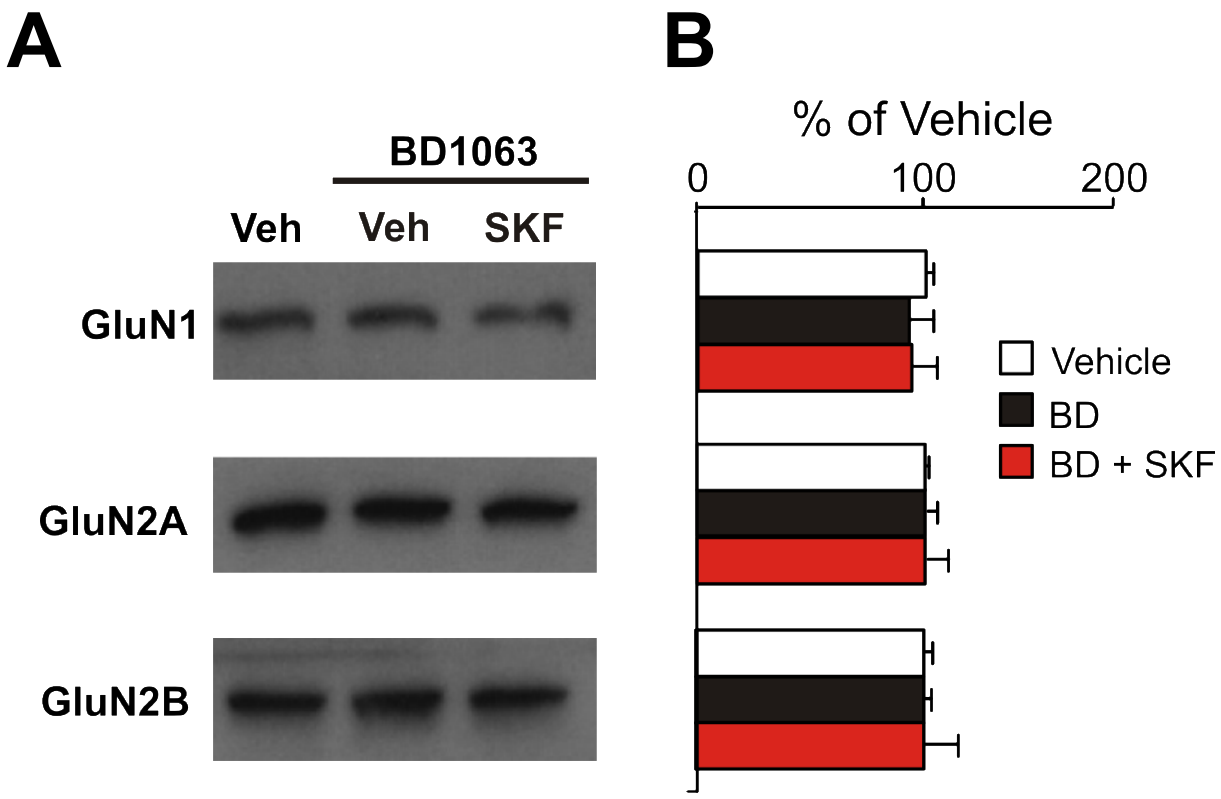

Figure 9: The increase observed in surface NMDARs following SKF administration is abolished in the presence of BD1063.

A representative WB showing that the σ-1R – activation dependent increase in surface NMDARs was abolished following pretreatment with the σ-1R antagonist BD1063, or BD, when compared to vehicle (Veh) (A–B). Bar graphs are mean ±SEM of at least 5 animals. This figure was modified from Pabba *et al*. (2014).

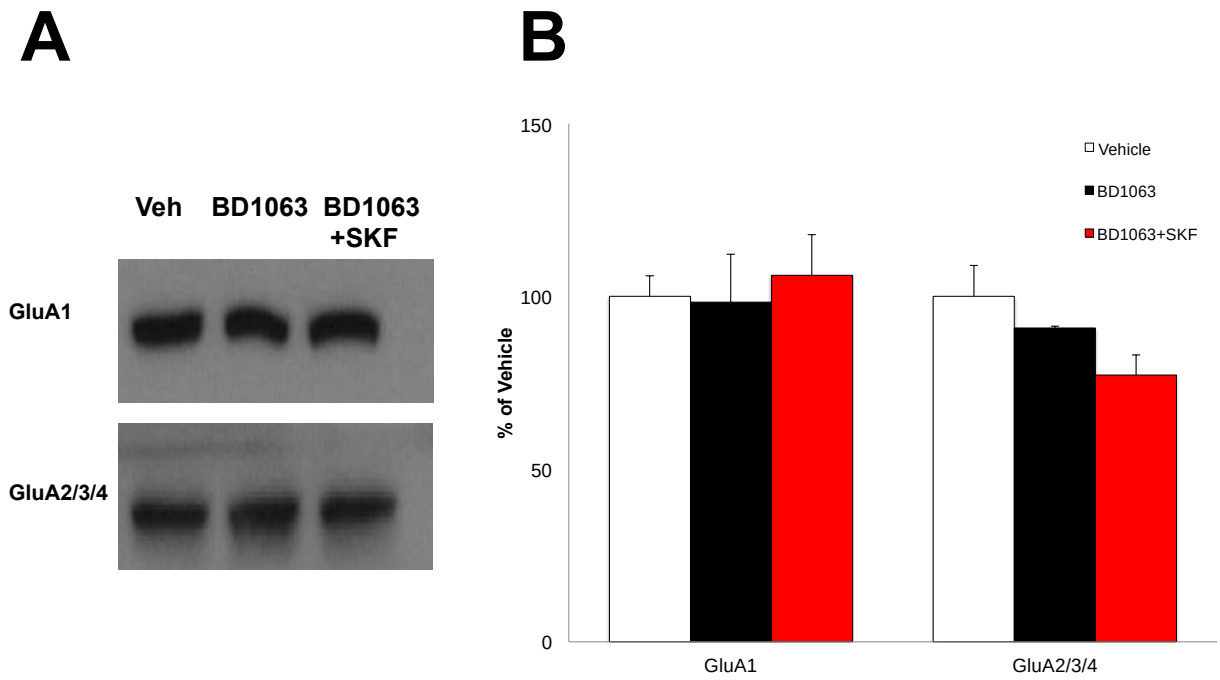

Figure 10: Surface AMPAR levels were not changed following SKF administration in the presence of BD1063.

A representative WB showing that surface levels of AMPAR GluA1, and GluA2/3/4 subunits were not changed following the administration of SKF in the presence of BD1063 (A–B) when compared to vehicle (Veh). Bar graphs are mean ±SEM of at least 5 animals.

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CONTRIBUTIONS OF COLLABORATORS

The data presented in this thesis is a collaboration between several authors. The experiments that I performed were the chronic administration of BD1063 *in vivo,* and the cell surface biotinylation. These results are represented by Figure 5, and Figures 8−10. The data from Figures 1−4, 6, and 7 was collected by Dr. Mohan Pabba and Dante Biscaro.