An investigation of sigma-1 receptor involvement in glutamatergic synaptic physiology, implications for Alzheimer's disease

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Abstract

The sigma-1 receptor (sig-1R) is a unique endoplasmic reticulum (ER) chaperone protein that interacts with a variety of voltage- and ligand-gated ion channels, which are components of an intricate system that regulates neuronal functioning. While there is an extensive body of knowledge pertaining to the sig-1R, many questions remain. The first question this thesis addresses is how the sig-1R modulates the functioning of the N-methyl-D-aspartate receptor (NMDAR). Using a heterologous expression system, I provide evidence that the mechanism of modulation is likely not a direct interaction between sig-1R and NMDAR and that this is not affected by the presence or absence of the membrane-associated guanylate kinases (MAGUK) protein PSD-95. The next question addressed investigates the impact of sig-1R absence on the synaptic physiology and action potential firing of CA1 pyramidal neurons. It was found that there is not a significant difference in these parameters, suggesting a non-essential role of the sig-1R under normal physiological conditions. The third topic covered in my studies explores the sig-1R KO mouse in the $A\beta_{25-35}$ infusion model of Alzheimer's disease (AD). Preliminary results suggest that there is a dysfunction in the action potential characteristics and after-hyperpolarization characteristics of challenged sig-1R KO mice. Overall my results provide the groundwork for future experiments that will lead to a better understanding of the sig-1R and its role in cellular and synaptic physiology.

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Abbreviations

Aβ	Amyloid Beta
ACSF	Artificial cerebrospinal fluid
AHP	After-hyperpolarizing potential
AP	Action potential
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
BiP	Binding immunoglobulin protein
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DHEA	Dehydroepiandrosterone
DTG	Ditolylguanidine
ER	Endoplasmic reticulum
GABA	Gamma-Aminobutyric acid
GFP	Green fluorescent protein
HEK	Human Embryonic Kidney
IP3R	Inositol 1,4,5-Triphosphate receptor
KO	Knockout
MAGUK	Membrane-associated guanylate kinase
MAM	Mitochondrial-associated membrane
mEPSC	miniature Excitatory Post-Synaptic Current
NMDAR	N-methyl-D-aspartate receptor
PKA	Protein Kinase A

PKC	Protein Kinase C
PPR	Paired-Pulse Ratio
PSD	Postsynaptic Density
PSD-93	Postsynaptic Density Protein 93
PSD-95	Postsynaptic Density Protein 95
PTZ	(+)-pentazocine
QX-314	Lidocaine N-ethyl bromide
SEM	Standard Error of the Mean
Sig-1R	Sigma-1 receptor
SK channels	Small conductance calcium-activated potassium channels
SKF	(+)-SKF-10,047
TTX	Tetrodotoxin
VGCC	Voltage-gated calcium channel
WT	Wild type
YFP	Yellow fluorescent protein

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Abstracts

1. **Kieran McCann**, Pabba M, Wong AY, Beique JC and Bergeron R. (2013) The Functional Modulation of the N-methy-D-aspartate Receptor (NMDAR) PSD-95 Complex *via* Sigma-1 Ligands. 4th Annual Brain Health Research Day. University of Ottawa

2. **Kieran McCann**, Pabba M, Wong AY, Ahlskog N, Hristova E, Biscaro D, Nassrallah W, Ngsee JK, Snyder M, Beique JC, and R. Bergeron (2014) NMDA Receptors Mediated Current Modulation *via* Sigma-1 Receptor Ligands in a Heterologous Expression System. CMM/NSC Research Day. University of Ottawa.

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Chapter 1: Introduction

1.1 Introduction: The sigma-1 receptor

The sigma-1 receptor (sig-1R) is a ligand-operated chaperone protein that is implicated in several neurodegenerative and neuropsychiatric disorders making it an exciting target for in depth investigation (Tsai, *et al*., 2014). The sig-1R has been shown to interact with and modulate the functioning of several membrane bound ion channels, such as voltage gated calcium channels (VGCC), potassium (K^+) channels, sodium (Na^+) channels and N-methyl-D-aspartate receptors (NMDARs). At the mitochondrial-associated-membrane (MAM) of the endoplasmic reticulum (ER), the sig-1R interacts with the ER chaperone protein Binding immunoglobulin protein (BiP) and the inositol-1,2,5-triphosphate receptor (IP3R) calcium (Ca^{2+}) channel (Hayashi & Su, 2007). The diverse role of the sig-1R in several cellular functions, such as action potential firing (AP), synaptic physiology and calcium homeostasis, make it a strong potential candidate for targeted therapeutic interventions in disease states such as Alzheimer's disease (AD) (Hayashi et al., 2011). AD is a devastating disease that manifests itself as a steady decline in cognitive function that eventually leads to the death of the individual. It is the leading form of dementia in people over age 60 (Sadigh-Eteghad *et al*., 2015). Classically characterized as the accumulation of amyloid-beta (Aβ) plaques, intracellular neurofibrillary tangles (NFT) and synapse loss, there are several hypotheses of the pathological process leading to AD. One underlying trend in the literature is the dysregulation of calcium homeostasis and the changes in the functioning of proteins responsible for Ca^{2+}homeostasis such as the ryanodine receptor and the NMDAR (Supnet *et al*., 2006, Danysz & Parsons, 2012). The indications that the sig-1R is responsive to changes in intracellular [Ca^{2+}] and has a diverse pharmacological profile make it an interesting and exciting avenue to better understand and potentially treat AD.

The objective of this thesis is to explore the unique functional characteristics of the sig-1R in a variety of models. The first investigation looks at how the sig-1R might be a modulator of the NMDAR in a heterologous expression model. The second goal of my thesis was to understand the role of the sig-1R in the synaptic physiology of CA1 pyramidal neurons by using the sig-1R knockout (KO) mouse. The final aim of my thesis begins to explore the role of the sig-1R in an animal model of AD.

1.2 History

When the sig-1R was first discovered it was misclassified as the sigma/opioid receptor, a subtype from the opioid receptor family. It was thought to be a subtype of opioid receptor because of the behavioral effects of SKF-10,047 (SKF) (N-allylnormetazocine) were distinct from those of the κ and μ opioid receptors (Martin et al., 1976). However, while studying the sigma/opioid receptor in 1982, Su determined that the receptor was insensitive to naloxone, an opioid receptor antagonist, and in 1988 the sig-1R was distinguished as separate from the opioid receptor family (Su, 1982; Su *et al*., 1988). Much of the initial conflict regarding the identity of the receptor type may be attributed to the lack of specificity of SKF as it has been shown to interact with $Na_V1.2/1.4$ channels, NMDARs and κ opioid receptors at higher concentrations (Vaupel, 1983, Su *et al.*, 2009, Gao *et al*., 2012). There are currently two subtypes of the sigma receptor, the sig-1R and sigma-2 receptor (sig-2R). The subtypes are distinguished by their different pharmacology in ligand binding assays. Hellewell et al. found the existence of the sig-2R in 1994. The sig-2R is by far the less studied and understood subtype of sigma receptor and is generally characterized as having less affinity for the classical sig-1R drugs (Bowen, 2000). The sig-2R remains uncloned and will not be the focus of this thesis (Hanner *et al*., 1996; Seth *et al*.,

1998; Mei & Pasternak, 2001). In contrast, the sig-1R has been cloned from guinea pig, human, mouse and rat (Hanner *et al*., 1995, Kekuda *et al*., 1996, Seth *et al*., 1997, Seth *et al*., 1998, Pan *et al*., 1998). The generation of the sig-1R-1 KO mouse provided a substantial advancement for sig-1R investigators (Langa *et al*., 2003). I utilize the sig-1R KO mouse extensively in chapters 4 and 5 and will discuss the details in future sections.

1.3 Structure & Localization

The sig-1R is a 25.3 kDa, 223 amino acid protein, with a proposed structure of two transmembrane domains (amino acids 11-29 and 91-109), and a third hydrophobic region (amino acids 176-194) on the C-terminus tail (**Figure 1A**) (Aydar *et al*., 2002, Su *et al*., 2010). Across the mammalian species for which the sig-1R has been cloned the amino acid sequence is 90% conserved and shares no homology to any other mammalian protein. It does share a similarity to the fungal protein C8-C7 sterol isomerase however it shows no sterol isomerase activity in yeast lacking the C8-C7 isomerase gene (Moebius *et al*., 1997, Hayashi & Su, 2005). The sig-1R binding sites are located at the N-terminus transmembrane domains, and have been named steroid binding domain like-1 (SBDLI) and steroid binding domain like-2 (SBDLII) (Pal *et al*., 2007, Pal *et al*., 2008, Su *et al*., 2010). The third hydrophobic region is on the C-terminus tail and is believed to contribute to the proposed binding pocket as well, indicated in the shaded area in **Figure 1A**. These binding sites display a broad range of affinities for a diverse selection of ligands (Hayashi & Su, 2005). Expression of the sig-1R extends beyond the CNS as it has been found in liver, placenta, immune tissue, kidney, heart, gonads, pancreas and lung (Seth *et al*., 1997, Zamanillo *et al*., 2000, Shamsal *et al*., 2001). Within the scale of the brain, the sig-1R is expressed broadly and found in the hippocampus, cortex, olfactory bulb, hypothalamus and

cerebellum. The temporal expression extends from young animals and is maintained in aged mice (Phan *et al*., 2003). The primary site where the sig-1R is found is at the MAM of the ER (Hayashi & Fujimoto, 2010). The N-terminus transmembrane domain contains a double arginine ER retention domain. In the inactive state, the sig-1R is bound to BiP. Upon activation of the sig-1R by one of its agonists or ER stress, it dissociates from BiP and is thought to carry out several different roles within the cell (**Figure 1B**). At lower doses of agonist it is believed that the sig-1R is mobilized at the MAM to stabilize the IP3R and facilitates calcium transport to the mitochondria, increasing cell metabolism. At higher doses of agonist (approximately 10 times Ki), the sig-1R is believed to translocate from the MAM to the plasma membrane and interact with numerous receptors and ion channels (Su *et al*., 2010). The unique structure and localization of the sig-1R, along with its broad distribution across the CNS and active mobility within neurons, make it a very curious and potentially bountiful subject for further investigation.

1.4 Pharmacology

The pharmacology profile of the sig-1R is broad and includes drug categories such as benzomorphans, antipsychotics, antidepressants, steroids and drugs of abuse (**Table 1**). Despite this drug diversity the sig-1R ligands generally maintain the N-substituted pharmacophore (C-N(R)-X-(Ph) (Fontanilla *et al*., 2009, Glennon, 2005). The designation of being an agonist or antagonist is not as obvious as it may seem. Classic antagonists such as BD1074 and BD 1063 were deemed antagonists because of their ability to attenuate the dystonia evoked by DTG (Matsumoto *et al*., 1991). Other agonists and antagonist were characterized by their ability to dissociate or prevent the disassociation of the sig-1R from BiP or increase the disassociation between ANK200 and IP3R-3 (Hayashi & Su, 2001). The suspected endogenous ligands of the

sig-1R include N,N-dimethyltryptamine (DMT), pregnenolone sulfate, dehydroepiandrosterone (DHEA), progesterone and other neurosteroids (Bergeron *et al*., 1996; Maurice *et al*., 1997; Monnet *et al*., 1995; Su *et al*., 1988, Fontanilla *et al*., 2009). The initial interest in the sig-1R was derived from its pharmacological profile and the effect these drugs had on animal behavior. The complex nature and somewhat vague definition of sig-1R agonists and antagonists require careful consideration when selecting them for experimental protocols and evaluating those results.

1.5 Function

Neuron physiology is dictated by several parameters such as cell type, receptor composition, and disease state. The ability of the sig-1R to modify the physiology of a cell is derived solely from its interactions with other proteins that control parameters of the cell such as action potential firing patterns, resting membrane potential, and pre and post synaptic strength. The sig-1R is known to interact with voltage- and ligand-gated, cation-permeable channels both at the plasma membrane and intracellularly. The sig-1R has been shown to modulate the functioning of voltage-gated channels, such as Ca^{2+} channels, K^{+} channels and Na^{+} channels, by both direct and indirect mechanisms. Ligand-gated ion channels that the sig-1R is known to modulate include the NMDAR and small conductance calcium-activated potassium channel (SK) (Martina *et al*., 2006). Intracellularly, the sig-1R co-localizes with the ER chaperone protein, BiP, and the IP3R. Evidence also suggests that it effects the functioning of the ryanodine receptor (Tagashira *et al*., 2013). The diverse array of sig-1R targets gives it several functional implications for the overall physiology of the neurons in which it resides. **Table 2** summarizes the known functional effects of sig-1R on ion channels found at the plasma membrane.

1.5.1 Ca^{2+} Channels

Ca^{2+} is one of the most strictly regulated ions and is vital for neuronal function and health. Therefore, changing Ca^{2+} dynamics within a cell can have drastic consequences. There are a variety of VGCCs found at the plasma membrane which include N-, L-, P/Q, and R types. The importance of Ca^{2+} channels relates to their role in both fast synaptic events and slower changes in signaling pathways (Lacinova, 2005). sig-1R ligands have been shown to inhibit all types of VGCC, but the context in which they elicit their effect is also important (Maurice *et al*., 2009). Zhang and Cuevas (2002) found that when the sig-1R agonist (+) pentazocine (PTZ) and ditolylguanidine (DTG) was applied it caused a depression in the Ca^{2+} currents of high-voltage-activated (HVA) Ca^{2+} channels. Sig-1R agonist also inhibited the rise in intracellular [Ca^{2+}] during ischemic events (Herrera *et al*., 2008). In the case of L-type, HVA, Ca^{2+} channels in retinal ganglion cells, sig-1R ligands inhibited Ca^{2+} influx and the two proteins were found to co-immunopercipitate (Tchedre *et al*., 2008). Conversely, in field recordings of CA1 hippocampus, sig-1R agonists potentiated long-term potentiation (LTP) in a L-type channel dependent manner (Sabeti *et al*., 2007) Similar varying results were found by Hayashi *et al*. (2000), in that sig-1R ligands have a different effect on cytosolic free Ca^{2+} depending on the source of Ca^{2+}. In the case of bradykinin-induced increase in intracellular [Ca^{2+}], sig-1R ligands potentiated the effect but after ER depletion, sig-1R ligands inhibited the depolarization-induced elevation in intracellular [Ca^{2+}]. There is also evidence that some the VGCC are inhibited by sig-1R ligands in a non-sig-1R manner as sig-1R ligands have been shown to inhibit KCl-induced increases in [Ca^{2+}] in both the wild type (WT) and KO genotype of sig-1R KO mice (Gonzalez *et al*., 2012). When it comes

to the role of the sig-1R on VGCC the physiological context is critical, as agonists have been shown to have varying effects.

The IP3R is an intercellular Ca^{2+} channel found at the ER and is responsible for transporting Ca^{2+} to the mitochondria, which is an important requirement for several cellular processes and cellular respiration (Bosanac *et al*., 2004). The consequences of IP3R dysfunction results in a decreased oxidative phosphorylation in the mitochondria and the activation of a cell stress response (Cardenas *et al*., 2010). In a seminal paper by Hayashi & Su (2007), the sig-1R was identified as a Ca^{2+} sensitive, ligand operated chaperone protein that facilitates and maintains Ca^{2+} transport from the ER to mitochondria at the MAM. The application of sig-1R agonists was also found to remove ankyrin, a regulating protein of the IP3R (Hayashi & Su, 2001, Wu & Bowen, 2008). The general consensus is the activation of sig-1R, by agonist or cell stress, assists Ca^{2+} transport to the mitochondria by the IP3R and therefore facilitates cell bioenergetics. It is reasonable to hypothesize that some of the neuroprotective properties of the sig-1R are directly related to the sig-1R's ability to counteract disease states where Ca^{2+} dysregulation is implicated.

1.5.2 K^+ Channels

The sig-1R has been shown to have functional and/or biochemical interactions with several subtypes of K^+ channels. The implications for these interactions are quite broad as K^+ channels are a critical component for maintaining resting membrane potential and action potential firing characteristics, a cellular parameter that I examine in chapters 3 and 4.

The general effect of sig-1R ligands is to inhibit K^+ channel currents. Soriani *et al*., (1999a,b) found that the sigma agonist PTZ and igmesine inhibited the transient and sustained

outward potassium current (I_A). In a particular study by Aydar *et al*., (2002) it was demonstrated that the sig-1R acts as a ligand-regulated auxiliary potassium channel subunit of the $K_V1.4$ channel. The $K_V1.2$ channels also form a complex with sig-1R that results in greater D-type K^+ current and neuronal hypoactivity (Zhang & Cuevas, 2005; Kourrich *et al*., 2012). In our own lab it was shown that selective sig-1R ligands inhibit SK channels, which leads to a greater NMDAR current (Martina *et al*., 2006). The potentiation of NMDAR currents *via* this sig-1R ligand application was also shown to enhance LTP in CA1 pyramidal neurons. Once again, the physiological context is important to consider when evaluating the sig-1R effect.

1.5.3 Na^+ Channels

Na^+ channels are responsible for the depolarization necessary for action potential firing and neuron excitability. The sig-1Rs effect on these channels has so far shown to be inhibitory. Persistent sodium currents are inhibited by the sig-1R agonist, DHEA sulfate (Cheng *et al*., 2008). Sig-1R agonists also increase action potential (AP) latency and shift the steady inactivation of Na^+ channels to a more negative potential, decreasing the population of channels able to gate and thereby, depressing AP firing (Cheng *et al*., 2008). The inhibitory effect was blocked in the presence of a G_i protein inhibitor and protein kinase C inhibitors (PKC), suggesting that there is a signaling pathway activated by sig-1R agonists (Maurice & Su, 2009). It has also been shown that the sigma agonist DTG inhibits $Na_V1.2$ in a sig-1R dependent pathway but that the agonist SKF directly inhibits $Na_V1.2$ and $Na_V1.4$ channels, independent of the presence of sig-1R, by blocking the ion channel of these proteins (Gao *et al*., 2012,). The overall effect of sig-1R inhibition of Na^+ channels results in decreased neuron excitability. The changes in AP firing are a large focus of chapters 3 and 4 and will be discussed further.

1.5.4 NMDAR

The NMDAR is subtype of glutamate gated ion channel along with α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) and kainite receptors. However, it is unique in that it requires glycine as a co-agonist. NMDARs are necessary for LTP, thought to be a cellular correlate of learning. At resting membrane potentials the channel pore is block by Mg^{2+}, which is relieved by depolarization of the cell, making the NMDAR a coincidence detector of presynaptic glutamate release and postsynaptic depolarization. Further, NMDARs are permeable to Na^{+}, K^{+} and, most notably, Ca^{2+} which is an important second messenger for several pathways (**Figure 2A**).

NMDARs assemble as di-heteromers or tri-heteromers comprised of two obligatory GluN1 subunits combined with two subunits from the GluN2 family (A-D) and/or the GluN3 family (A-B) (Paoletti *et al.*, 2013). Gating of the NMDAR occurs upon the simultaneous binding of glutamate to the GluN2 subunit and glycine or D-Serine to the GluN1 and/or GluN3 subunit. It is primarily the GluN2 and GluN3 subunits that give the receptors their distinct characteristic gating properties. For example, GluN2B-containing NMDARs have a much slower decay kinetic, compared to GluN2A (Paoletti *et al.*, 2013). GluN2A and GluN2B have similar conductance of Ca^{2+} however, the slower decay kinetics of GluN2B is important as it allows a greater amount of Ca^{2+} to enter the cell upon stimulation. Therefore, changes to NMDAR subunit composition has a large impact on Ca^{2+} related signaling cascades and cell physiology, which leads to further effects such as gene transcription and excitotoxicity.

The postsynaptic membrane is a complex and diverse neighborhood in which the NMDAR resides. In this postsynaptic zone, NMDAR interacts with scaffolding proteins that can alter its location, functionality, and which signaling molecules are in close proximity (Chen *et*

al., 2011). One such protein is post-synaptic density-95 (PSD-95), a member of the membrane associated guanylate kinase (MAGUK) protein family, it along with PSD-93 and SAP-102, provide structural scaffolding at synapses and act as anchor points for several ions channels and receptors. PSD-95 is richly expressed in excitatory glutamatergic synapses (Beique & Andrade, 2003; Bard & Groc, 2011). The protein consists of three PDZ domains to which the NMDAR can anchor. The GluN2A and GluN2B subunits of NMDAR bind to the first two PDZ domains of PSD-95 (Sanz-Clemente, Nicoll & Roche, 2012) (**Figure 2A**). These domains are also responsible for binding K^+ channels and nitric oxide synthase (Migaud *et al*. 1998). PSD-95 also contains a Src-homolog 3 (SH3) domain and a guanylate kinase (GK)-homology domain. Several studies have explored the role of PSD-95 at excitatory synapses and shave shown it to be a key modulator of synaptic physiology.

The effect of sigma-1R ligands on NMDARs has been investigated from both a functional and biochemical perspective and will be discussed in more detail in chapter 2. Despite these investigations, the interaction between the sig-1R and the NMDAR and the implications of this interaction on functionality has not been conclusively determined. There is a strong interest in the modulators of NMDARs as it is the excitatory glutamatergic ion channel critical for learning and memory and is proposed to be altered in many disease states (Paoletti *et al*., 2013). If the sig-1R is able to modulate the functioning of the NMDA receptor it could have implications for numerous pathologies. Recently published investigations by Pabba *et al*., 2014, showed that the intraperitoneal injection of SKF, PTZ and PRE-084 increased the protein expression levels of GluN2A, GluN2B and PSD-95. It has also been demonstrated *via* in situ proximity ligation assays, that the sig-1R directly interacts with the GluN1 subunit and not the GluN2A subunit of the NMDA receptor (Balasuriya *et al*., 2013). Further, in a paper published

by Martina *et al*., (2007) it was shown that the bath application of the sig-1R agonist PTZ on CA1 hippocampal neurons potentiated the evoked, pharmacologically isolated, NMDA current. Although, as previously mentioned, the explanation of this phenomenon was attributed, in part, due to the inhibition of SK channels by the sig-1R, which is known to shunt Ca^{2+} conductance to the NMDAR. The effect of sig-1R ligands on NMDAR function was well established in the 1990's as demonstrated in the potentiation of NMDA induced firing rate in the CA3 of rat hippocampus in response to intravenous administration of sig-1R ligands (Debonnel *et al*, 1996). Despite extensive research, how exactly the sig-1R interacts with the NMDAR is still unclear. Given the evidence for the functional potentiation and enhanced expression of NMDARs, looking deeper into this response would provide greater understanding of how the sig-1R might interact with this crucial glutamatergic ion channel.

1.6 Working Hypotheses

The open questions of my thesis are admittedly broad. The overall intent was to better understand the role of the sig-1R from an electrophysiological perspective. The scope in which I investigated the sig-1R included, the potentiation of NMDAR mediated currents by sig-1R ligands, the impact of sig-1R absence on the basic electrophysiological properties of synaptic transmission in CA1 pyramidal neurons and the response of sig-1R KO mice when challenged by Aβ infusion in from an electrophysiological perspective.

My first goal was to investigate the potentiating effect of sig-1R ligands on NMDAR currents. The potentiation of NMDAR mediated currents *via* sig-1R ligands has been well demonstrated but is not incompletely understood. Using a heterologous expression system I attempted to dissect the functional effect of the sig-1R ligand, PRE-084, on the specific GluN2A

and GluN2B NMDARs. The role of PSD-95 in this potentiating effect was also investigated because of the biochemical evidence that in is incorporated into a sig-1R, NMDAR complex.

My second goal was to evaluate what the consequences of sig-1R loss are for synaptic physiology. The development of the sig-1R KO mouse is an excellent tool for better understanding of how sig-1R is involved in basic synaptic transmission. Few reports have been published on the electrophysiological properties of these mice and the KO mice have been shown to have a subtle, sex specific phenotype. My initial screening of these mice focused on the synaptic physiology of CA1 pyramidal neurons in adult male KO mice.

The third goal of my thesis was to investigate the electrophysiological impact of $A\beta_{25-35}$ infusion on the CA1 pyramidal neuron in wild type and sig-1R KO mice. The sig-1R has been implicated in the pathophysiology of AD but has only been mildly investigated in animal models of AD. The goal of my preliminary experiments was to probe for changes in action potential firing characteristics. The hope of these preliminary results is that they will guide further investigations into the pathophysiology of AD and if the action of the sig-1R in this disease state.

Chapter 2: Materials and Methods

2.1 HEK293 Cell Preparation

HEK293 cells were cultured at 37°C in a 5% CO2:95% air humidified incubator. Culture media was comprised of MEM with 10% FBS 1% Glutamax and 1% penicillin streptomycin. Cells were passaged every 3-4 days via trypsination and passaged a maximum of 25 times. Cells were seeded at approximately 30% confluency on 15mm coverslips prior to transfecting. Cells were transiently transfected 24 hours after splitting using the transfection reagent Lippofectamine 2000 and equimolar amounts of cDNA for GluN1, GluN2A, GluN2B, PSD-95-GFP, σ-1-YFP or σ-1-mCherry, depending on the desired genetic profile. The ratio of transfection reagent to cDNA was 1.5μg:1 μg (as per recommendation), 0.5 μg cDNA per well. Transfected cells were ready for whole-cell patch clamp electrophysiology 24-48 hours post transfection (**Figure 5**). This protocol produced transfection success rates from 50-70% and co-transfection rates were high as well. Co-transfection is confirmed by the presence of fluorescent proteins used, indicating sig-1R and PSD-95 and the ability to evoke a response the glutamate and glycine, indicating GluN1 and GluN2A/B. Transfected HEK293 cells were cultured in 200 μM DL-APV (glutamate site antagonist) and 50 μM 5,7-dichlorokynurenic acid (glycine site antagonist) to prevent excitotoxic cell death.

2.2 HEK293 Cell Data Recording and Analysis

Data recording was performed via whole-cell patch clamp recordings techniques in voltage-clamp mode using borosilicate micropipettes filled with: 115 mM NaCl, 10 mM NaF, 5 mM HEPES, 5 mM BAPTA, 0.5 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 10 mM ATP (pH 7.3 osmolarity 295). Cover slips were placed in a submerged recording chamber and superfused with

HEK293 cell recording solution containing: 150 mM NaCl, 10 mM HEPES, 3 mM KCl and 2 mM $CaCl_2$ (pH 7.2 with NaOH)

Once access was gained to the cell, NMDAR mediated currents were evoked via picospritzer pipette loaded with glutamate [10mM] and glycine [100uM], the co-agonists for NMDA receptors, placed approximately 5-15 uM from the cell (**Figure 6D**). HEK293 cells were selected based upon the indication that they are transfected via fluorescent proteins, if they appear healthy based upon their morphology and that they are not too confluent with neighboring cells (**Figure 6D**). NMDAR currents will be evoked by a brief puff (5-40 msec) of NMDA receptor agonists from the picospritzer at a frequency of 0.03-0.05 Hz. Flow rate of the recording solution was controlled *via* drip set at 1-2 mL per minute. Traces were recorded for later analysis. The design of the experiment contained three phases, first a period of control followed by a period of application of the treatment and a wash out period. The primary data gathered from these experiments was the peak amplitude of the NMDAR current. The primary sig-1R agonist investigated was PRE-084.

2.3 Animals

Male C57BL/6j x 129s/Sv mixed background WT and KO mice were bread in house. Animals were housed in a step-down isolation unit with a 12 hour light/dark cycle and free access to food and water. Genotyping was confirmed by PCR. Animals were used ranged from 6-10 weeks of age to 6 months in age. For animals treated with $A\beta_{25-35}$, animals were transferred to a surgical suite where they were injected i.c.v with 9 nM $A\beta_{25-35}$ and allowed to recover for one to two weeks.

2.4 Slice Preparation

In accordance with the guidelines and approved methods of the University of Ottawa Animal Care and Veterinary Services and the Canadian Council of Animal Care, prior to decapitation, the animals were anaesthetized using an isofluorane vaporizer (Stoelting, Wood Dale, IL, USA) After decapitation the brain was quickly removed and submerged a slush (<4°C) of choline chloride cutting solution containing: 119 mM Choline Chloride, 2.5 mM KCl, 4.3 mM $MgSO_4$-$7H_20$, 1 mM $CaCl_2$-2H2O, 1 mM NaH_2PO_4, 1.3 mM Na-Ascorbate, 11 mM Glucose, 26.2 mM $NaHCO_3$ (pH 7.3, osmolarity 295 mOsm, 95% O_2 – 5% CO_2). After cooling for 1 minute, the brain was blocked and mounted in the coronal plane and 300 μm hippocampus-containing slices were cut with a vibrating microtome, (Leica VT 1000S, Germany). Slices were allowed to recover initially in an oxygenated chamber of artificial cerebral spinal fluid (ACSF) containing: 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$-$7H_2O$, 2.5 mM CaCl2-2H2O, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose, at 37°C and allowed to equilibrate to room temperature over one hour. Cells were transferred to a submerged recording chamber post recovery, and superfused (2-4 ml/min) with oxygenated ACSF at room temperature with the exception of mEPSC recordings being done at 30°C with the use of a perfusion chamber heater.

In the interest of maintaining the best possible cell quality for cell patching in the 6 month old mice, a cardiac perfusion technique was used. Once deeply anaesthetized, the parietal and thoracic cavity was opened and the animal was perfused with 10 mL of ice cold, choline chloride cutting solution (see above) *via* the left ventricle of the heart. The rapid cooling of the brain within the skull has been found to produce optimal slices for recording in aged animals. After perfusion the brain is quickly removed and mounted for slicing as previously discussed

2.5 Data Recording

For experiments performed in voltage-clamp mode, whole-cell patch-clamp recordings were obtained with borosilicate micropipettes filled with either a K-gluconate-based intracellular solution containing: 134.9983 mM K-gluconate, 6.9752 mM KCl, 10 mM *N*-2-hydroxy-ethylpiperazine-*N*-2-ethanesulphonic acid (HEPES), 4 mM Mg-ATP, 0.4014 mM GTP-tris, 10 mM Sodium Phosphocreatine or a Cs-methanesulfonate-based intracellular solution containing: 115 mM Caesium Methane-Sulfonate, 0.4 mM EGTA, 5 mM TEA-Cl, 6.67 mM NaCl, 20 mM HEPES, 4 mM ATP (Mg salt), 0.5 mM GTP, 10 mM Sodium Phosphocreatine, 5 mM QX-314-bromide. The internal solution's pH and osmolarity were adjusted to 7.3 and 290 mOsm, respectively. The resistance at the pipette tip ranged from 4-7 MΩ and an access resistance of < 25 MΩ was considered acceptable. Access resistance and input resistance were monitored throughout the experiment by a 200 msec long, -5mV step in voltage clamp mode or a -25mV step in current-clamp mode. Depending on the experimental protocol a variety of blocking drugs were used which include, sodium currents were blocked by adding 0.5 mM tetrodotoxin (TTX) for sodium currents, 100 μM picrotoxin for $GABA_A$, to the ACSF.

For experiments performed in current-clamp experiments mode, slices were perfused with normal ACSF and recordings were obtained with electrodes filled with recording electrodes were filled with a K-gluconate-based intracellular solution (see above). K-gluconate-based intracellular solution was chosen for recording the action potential as it allows the different ionic conductance's (Na^+, K^+ and Ca^{2+}) of hippocampus neurons. No blocking drugs were used for current clamp recordings.

2.6 Data Analysis

Recordings were obtained with a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA) telegraphed to a Digidata 1322A 16-BIT data acqusition system (Axon Instruments, Foster City, CA, USA). HEK293 cells were visualized using a Leica DM LFSA using contrast microscopy and infrared video microscopy. Hippocampal neurons were visualized using a Zeiss LSM 510 using differential interference contrast III (DIC-III) and infrared video microscopy.

Data were collected using pCLAMP 9.2 software (Axon Instrument, Foster City, CA, USA). Analysis was performed *off*-line with the software Clampfit 10.3 (Axon Instrument, Foster City, CA, USA). Differences in HEK293 cell recordings were determined by Student's *t-test* between the 5 min and 15 min time points as well as two-way repeated measures ANOVA comparing treatment *versus* sham groups. Differences between wild-type and knockout populations was determined with a Student's *t*-test ($P<0.05$) and two-way repeated measures ANOVA *N* indicates the number of cells in each group and all values are expressed as means ± SEM.

2.7 Drugs

D,L-AP5, 5,7-dichlorokynurenic acid, NBQX di-sodium salt, and PRE-084 were purchased from Abcam (Cambridge, MA). Tetrodotoxin (TTX) was purchased from Tocris Cookson (Ellisville, MO). D-APV and (+) pentazocine (Sigma-Aldrich (MO, USA).

Chapter 3: Functional effect of sig-1R activation on NMDAR current

3.1: Introduction

Our lab has a long history exploring interactions between the sig-1R and the NMDAR. First observed by Monnet *et al*. (1990), and explored further by Debonnel et al (1996a,b) and Dr Bergeron the functional effect of sig-1R ligands on NMDAR currents has been well characterized. Dr. Bergeron's research in the 1990's showed that sig-1R activation, by the ligands PTZ, DTG and JO-1784, selectively potentiated the NMDAR mediated field recordings in rat CA3 hippocampus (**Figure 3A**) (Bergeron *et al*., 1995, Debonnel *et al*., 1996). In my own experience, I have generated positive preliminary data showing the potentiation of isolated NMDAR current in the CA1 of mouse hippocampus with PTZ (**Figure 3C**). As previously mentioned, our lab has also shown that a sig-1R-SK channel mechanism is partly responsible for the potentiation of NMDARs (**Figure 3B**) (Martina *et al*., 2007). Despite these well-characterized effects of sig-1R ligands on NMDARs the exact mechanism remains elusive. Potential explanations for this effect are an increase in the number of NMDAR at the plasma membrane by redistribution of receptors to the surface or the stimulation of protein synthesis. Another possibility is that there is a change in the functional characteristics of the NMDAR.

Our lab had recently publishing a paper demonstrating that an intraperitoneal injection of the sig-1R agonists SKF, PTZ and PRE-084 significantly increased the levels of GluN2A, GluN2B and PSD-95 in the synaptosomal fraction of the hippocampus when compared to control animals (**Figure 4A-D)** (Pabba *et al*., 2014). The synaptosomal fraction is obtained by differential centrifugation and provides an acute representation of synaptic biochemistry. The results of this experiment demonstrated that there is an increase in the population of NMDARs in the synaptosomal membrane fraction following treatment with a sig-1R agonist. Further

investigations using co-immunoprecipitation techniques demonstrated that there is an interaction between NMDARs, the sig-1R and PSD-95, which occurs with and without sig-1R agonist treatment (**Figure 4E**). Based upon the biochemical evidence showing that treatment with sig-1R agonists causes an increase in NMDAR subunits at the synapse and that sig-1R biochemically interact with NMDARs and PSD-95 it is prudent to ask what impact this relationship has on functional aspects of NMDARs. There is also a discrepancy in the time scale between the functional and biochemical effect of sig-1R ligands. In electrophysiological recordings the potentiation of NMDAR currents is fairly quick (10-20 min) whereas the biochemical effect is not observed until 90 minutes after sig-1R treatment, suggesting a multimodal mechanism of sig-1R activation.

In this chapter, I explore how sig-1R ligands modulate NMDAR function. While published data clearly demonstrate that sig-1R alter NMDAR expression and function, it is unknown if these effects are due to direct actions on the NMDAR itself or are secondary effects from sig-1R acting on other proteins and signaling cascades. Furthermore, if sig-1R is acting directly on NMDARs, it is unknown which receptor subtype(s) are affected. Because native tissue contains multiple NMDAR subtypes and many other proteins known to be effected by sig-1R, it is necessary to utilize a heterologous system, which will allow greater experimental control. A heterologous expression system allows one to deconstruct the complexity of native tissue's genetic profile so that sig-1R effects on NMDAR currents and the role of PSD-95 can be more clearly demonstrated. HEK293 cells will be utilized, as they will readily translate GluN1, GluN2A, GluN2B and PSD-95 cDNA (Thomas & Smart, 2005). The sig-1R is also endogenously expressed in HEK293 cells (**Figure 6C**). The GluN2A and GluN2B-containing NMDAR subtypes will be investigated individually, in the presence or absence of PSD-95.

These experiments will further elucidate if and how sig-1R modulates NMDARs and will provide the groundwork for a larger investigation into sig-1R effects on synaptic physiology.

3.2 Results

The goal of these experiments was to determine the effect of sig-1R activation, by the agoinst PRE-084, on specific NMDAR subtypes. HEK293 cells were transfected with GluN1, and GluN2A or GluN2B and currents were examined using whole cell patch clamp electrophysiology. HEK293 cells were selected for patching on the basis of their healthy morphology and being relatively isolated from neighboring cells (**Figure 6D**). Successful transfection was determined by the presence of m-Cherry, GFP or YFP fluorescence, depending on the genetic profile of the transfection, and being able to exhibit a current evoked by glutamate and glycine (**Figure 6A, B, E**). The NMDAR subtypes of interest both exhibited decay kinetics stereotypical of their subtype, in that the GluN2A NMDARs decayed much faster than the GluN2B subtype (**Figure 7A**). However, it should be noted that the decay tau of the NMDARs in HEK293 cell is much greater than neuronal NMDARs because in the HEK293 cell model receptor deactivation is dependent on the glycine and glutamate being washed away, dependent on flow rate of the perfusion system, whereas in acute tissue slices, reuptake and enzymatic degradation removal gating agonists much faster (**Figure 7B**). Preliminary results also indicated that the model was sensitive to changes in the flow rate as a result of changes in the pressure head of the feeder tube or switching between feeder tubes (**Figure 7C**) and therefore strict monitoring of the pressure head and flow rate is necessary.

The treatment dose of 200 nM PRE-084 was chosen because it is described as a new generation of highly specific sig-1R agonists (Maurice *et al*., 1994). Previously publish studies

have used a variety of sig-1R agonists such as SKF, PTZ and PRE-084. The dosage used in studies ranged from 20 nM to 100 μM. My choice of 200 nM PRE-084 was decided upon because PRE-084 is reported to have a five times greater affinity for sig-1R than the classic agonist PTZ which is commonly used at 1 μM. Bergeron *et al* (1995) has also reported on the biphasic effect of sig-1R ligands PTZ and DTG, where low doses of agonist potentiate the NMDAR, but at higher doses the NMDAR is inhibited. Unfortunately, PTZ is also a drug of abuse and strictly regulated by Health Canada, which further limits its usefulness. PRE-084 is advantageous to PTZ because of its stability in solution where as PTZ has a short shelf life and degrades when exposed to light. The classic sigma ligand SKF was eliminated because it has been reported to inhibit the PCP site of NMDARs. I briefly explored SKF as an agonist but confirmed it to have an NMDAR antagonist effect by reducing the NMDAR current amplitude by approximately 70% ($p=0.0008$, $n=3$) at 1 μM (**Figure 8C**). Inset representative traces indicate the control, treatment and post treatment period. The antagonistic properties of SKF and other sigma drugs is a well describe and a common problem for sig-1R research which is likely what prompted the development of newer sigma-1 drugs such as PRE-084 (Fletcher *et al*., 1995).

Evoked currents were allowed to stabilize and then recorded for 5 minutes of stable baseline. The sig-1R ligand PRE-084 (200 nM in HEK293 ringer) was applied for a 10 minute period while continuously recording. After the ten minute treatment period the "treatment" HEK293 ringer was exchanged with normal HEK293 ringer. A "Sham" control treatment was also used where the same ringer switch occurred but no drug was present at any time. Statistical significance was determined by *t*-test between the 5 minute mark of the control period and the 10 minute mark (15 minute mark of total time) of the treatment period as well as two-way repeated measures ANOVA comparing the treatment and "sham" in each condition.

I first investigated GluN2A containing NMDARs by transfecting HEK293 cells with equimolar amounts of GluN1, GluN2A, sig-1-YFP and m-Cherry. Over the course of the drug treatment no significant difference was observed in the peak current amplitude (p=0.6989, n=7) (**Figure 8A**). These results are similar to the "sham" treated GluN2A NMDAR cells (p=0.8001, n=12) (**Figure 8A**). Two way repeated measures ANOVA comparison between treatment and sham were also not different (F=1.187, p=0.2734). These results indicate that, in the case of GluN2A type NMDARs alone, the sig-1R agonist is unable to potentiate the current amplitude. These results suggest that a key component of the mechanism required for sig-1R ligands to potentiate NMDAR mediated currents is lacking. As previously mentioned, the findings of Pabba *et al*. 2014, suggest that the up regulation of PSD-95 is also observed with intraperitoneal treatment of sig-1R ligands. To investigate the role of PSD-95, HEK293 cells were transfected with equimolar amounts of GluN1, GluN2A, PSD-95-GFP and sig-1R-mCherry. Under the same treatment paradigm the PSD-95 containing cells also failed to elicit a potentiating response to PRE-084 (200 nM) (p=0.8017, n=5) (**Figure 8B**). "Sham" treatment of these cells also shows a stable current amplitude over the course of the experiment, indicating the absence of an effect (p=0.4656, n=10) (**Figure 8B**). Two way repeated measures ANOVA comparison between treatment and sham were also not different (F=0.3841, p=0.9983). Inset traces of the PRE-084 treated cells are shown in **Figure 8A-B** representing the control, treatment and post-treatment time points. There was a range in the amplitude of GluN2A NMDAR currents (approx. 50 pA – 1000 pA) which varied depending on the transfection. Once the current was stabilized, there was no difference noticed between large or small currents in relation to the treatment.

GluN2B NMDARs were the next subtype investigated using the same transfection protocol and experimental design. HEK293 cells were transfected with GluN1, GluN2B, sig-1R-

YFP and m-Cherry. When GluN2B type NMDARs were treated with 200 nM PRE-084, no potentiating effect was observed (p=0.8017, n=8) (**Figure 9A**). The "sham" treated GluN2B NMDARs did not respond with any noticeable effect as well (p=0.0776, n=8) (**Figure 9**A). Two way repeated measures ANOVA comparison between treatment and sham were also not different (F=0.4321, p=0.9954). Similarly, when PSD-95 was included in the transfection with GluN2B there was no potentiating effect observed however, there was approximately a 20% rundown in the current (p=0.0056, n=5) (**Figure 9B**). A similar, but not significant rundown was observed in the "sham" treated GluN2B NMDARs with PSD-95 (p=0.1223, n=8) (**Figure 9B**). Two way repeated measures ANOVA comparison between treatment and sham were also not different (F=0.9359, p=0.5591). Inset traces of the PRE-084 treated cells are shown in **Figure 9A-B** representing the control, treatment and post-treatment time points. There was a significant current "rundown" ($p<0.0001$) over time in all the GluN2B conditions. I believe this to be an artifact of the picospritzer model and not a sig-1R effect. The range of current amplitudes for GluN2B NMDARs was approx. 50 pA – 500 pA and again, there was no difference noticed between large or small currents in relation to the treatment.

Overall the sig-1R agonist PRE-084 was unable to elicit a potentiating response in GluN2A or GluN2B NMDARs. The presence of PSD-95 did not have an observable effect either, with the exception of showing a greater rundown of the GluN2B NMDAR mediated current. At first pass these results are suggestive that the mechanism by which sig-1R ligands potentiates NDMAR currents in native tissue is not a direct ligand-sig-1R-NMDAR interaction and other key components are lacking in the heterologous expression system.

3.3 Discussion

Previous work in our and other laboratories demonstrates that sig-1R agonists potentiate NMDAR mediated current. However, in my experiments, for both GluN2A and GluN2B NMDAR subtypes, I was unable to elicit a potentiating effect with the sig-1R agonist PRE-084. The presence or absence of PSD-95 did not have any effect on evoked currents. Interpretation of these results is challenging given the absence of an effect but some conclusions may be drawn.

The first thing I called into question was the dose of sig-1R agonist (200 nM PRE-084) I used for my experiments. As previously discussed, the choice of this concentration was well reasoned based upon previous studies. To confirm that my choice of agonist was appropriate, I treated the GluN2A and GluN2B transfected HEK293 cells with and without PSD-95 with 1 μM PRE-084. The fivefold higher concentration of agonist also failed to elicit a potentiation of the NMDAR current and the current amplitude was not significantly different between time points 5 and 15 in any of the groups (**Figure 10**). I also briefly explored lower doses of PRE-084 (20 nM) but got similar negative results as previously tested doses (data not shown). I feel PRE-084 is an appropriate agonist given its high specificity for sig-1R and minimal interaction with off target sites. The question remains, what is necessary for sig-1Rs to modulate NMDARs?

One possibility is that activation of sig-1Rs by a highly specific ligand does not directly modulate the functioning of NMDARs and involves a second messenger system. NMDARs have long cytoplasmic tails that are subject to both serine and tyrosine phosphorylation (Wang *et al*., 2014). Changes to the phosphorylation state of the receptor can change its trafficking and gating characteristics, resulting in a modulation of current amplitude. For example the phosphorylation of S896 and S897 increases the surface expression of NMDARs (Scott *et al*., 2001). Jones and Leonard (2005) showed that the PKC mediated phosphorylation of S1291 and S1312 in GluN2A

potentiated the NMDAR current. As previously mentioned the known interactions with sig-1R include both direct channel interactions and modulatory effects through second messenger pathways such as PKC and or calcium/calmodulin-dependent protein kinase (CaMK) II and IV (Hayashi *et al*., 2005).

In the case of the sig-1R with PKC and CaMKII , it has been shown that the agonist DHEA rescued GluN1 phosphorylation levels in the CA1 of olfactory bulbectomized mice and also rescued the behavioral deficits of these mice (Moriguchi *et al*., 2011). PKC phosphorylation of GluN1 was also induced by sig-1R ligands in animal models of NMDA-induced pain perception (Kim *et al*., 2008).

Though HEK293 cells have a fairly basic genetic makeup they do contain several endogenous proteins of interest such as PKC and PKA (Thomas & Smart, 2005). As the role of phosphorylating proteins may be involved in how the sig-1R modulates NMDAR functioning I briefly explored this potential avenue. To probe for PKC activity in my heterologous expression system I attempted to elicit a response in GluN2B NMDARs using a K^+Gluconate recording internal. My original HEK293 recording internal contained NaF, an inhibitor of PKC. If the NaF in my original recording internal solution was inhibiting the phosphorylation of the NDMAR subunits and preventing the modulatory effect I was attempting to elicit this new internal could produce a different result. However, the different internal recording solution did not result in a potentiating effect of cells transfected with GluN2B NDMARs in the absence of PSD-95 and treated with 200 nM PRE-084 ($p=0.1682$, $n=4$) (**Figure 9C**). The results of this secondary experiment were similar to the initial results I obtained. I cannot however say if there was a change in phosphorylation of the NMDAR subunits as demonstrated in the previously mentioned papers. This one experiment is also only looking at one type of NMDAR and PSD-95 was not

present. If I were to revisit these experiments in the future for further investigation I would take a broader approach and look to evaluate the potential role of second messenger systems in the sig-1R ligand mediated potentiation of NMDARs.

Chapter 4 Sigma-1R KO Characterization

4.1 Introduction

Neuron-to-neuron communication is an essential quality of healthy brain functioning. Communication between neurons occurs at synapses. Several neurological disorders, such as AD, exhibit synaptic dysfunction. Synaptic physiology pertains to the functional characteristics of this neuron-to-neuron communication. Synapses are comprised of a presynaptic terminal which, upon stimulation by an action potential, releases neurotransmitter vesicles that bind to and activate receptors on the postsynaptic terminal. The two main neurotransmitters are *gamma*-Aminobutyric acid (GABA) and glutamate. My work focuses on the glutamate system. The glutamatergic system is the major excitatory neurotransmitter in the brain and stimulates NMDA, AMPA and kainate subtypes of receptors. In chapter 2, I focused on how the sig-1R interacts with the NMDAR in isolation. In this chapter, I investigate if or how the sig-1R affects the action potential characteristics and synaptic physiology of CA1 pyramidal neurons. For this, I make use of the sig-1R KO mouse.

Langa *et al*. (2003) generated the sig-1R KO mouse by homologous recombination techniques in mouse embryonic stem cells with the goal of better understanding the in vivo role of the sig-1R. The total knockout was confirmed with southern blot, northern blot and western blot as well as in situ hybridization (**Figure 11A-D**). Probing the sig-1R KO with the specific ligand, [3H](+)pentazocine, was devoid of any signal however, when probed with [+3]DTG, a non-specific sigma ligand, binding sites were observed suggesting that the sig-2R is encoded on a different gene and not a splice variant of the sig-1R gene. Despite its ubiquitous expression throughout the brain, liver, kidney, heart and other organs, it was found that the sig-1R KO mice

did not have an overt phenotype. The KO mice exhibited normal metabolic, feeding, nesting, lactation, maternal care and heterozygous breeding pairs produced offspring of expected Mendelian frequency. A notable characteristic is that the KO mice were significantly more resistant to the hypermotility response of SKF, an often-used protocol for inducing an animal model of psychosis. The behavioral profile of the sig-1R knockout mice has been characterized in open-field behavior tests, water-maze, Y-maze spontaneous alternation, step-through passive avoidance, forced swimming, and the elevated plus-maze (Chevallier et al., 2011). The overall findings of this study were that male sig-1R KO mice exhibited a phenotype of increased anxiety and depression whereas the female sig-1R knockout mice displayed a phenotype of spatial memory impairment. Interestingly, the spatial memory impairment of female knockout mice was attenuated when they were treated with 17β-estradiol. The implications of this study demonstrated that the actions of the sig-1R has some sex specific roles in mice which may be due to the fact that steroid hormones can bind to the sig-1R. It was also found that the KO mice exhibit decreased swimming and increased immobility time in the forced swim test (Sabino *et al*., 2009) Pharmacological intervention for the male mice showed a decrease in immobility when treated with tri-cyclic antidepressant therapy and selective serotonin reuptake inhibitor therapy but the sig-1R KO did not respond to the antidepressant igmesine, a known sig-1R agonist.

Additionally, the sig-1R KO mouse was less responsive to non-acute, formalin-induced pain, suggesting a role for sig-1R in pain (Cendan *et al*., 2005). In regards to neurogenesis, KO mice exhibit greater proliferation but decreased density and number of surviving newborn neurons in the dentate gyrus (Sha *et al*., 2013). These differences were rescued in the KO mice with the application of a selective GluN2B NMDAR agonist. In the SOD1*G93A mouse model

of ALS it was found that knocking out the sig-1R reduced life span and caused an increase in the excitability of motor neurons (Mavlyutov *et al*., 2013). There is a trend in the sig-1R field in that under normal physiological conditions the sig-1R does not play a big role in normal cell functioning. However, when the physiology is challenged the role of the sig-1R becomes more apparent.

Despite this extensive work that has been done with the sig-1R KO mice, little is known about any changes to synaptic functioning and physiology. In this chapter, I characterize the hippocampal CA3-CA1 synaptic functioning in male sig-1 KO mice using whole cell recording electrophysiology techniques in acute slice preparations. This synapse is one of the most well studied synapses in the brain, making it an ideal location to probe for changes following loss of sig-1R. The behavioral characteristics such as impaired spatial memory seen in the KO mice could be attributed to changes in hippocampal function. Further, the hippocampus is an essential structure for learning and is one of the first structures affected in AD. Thus, the experiments in this chapter will provide a solid foundation for our later work examining how loss of sig-1R contributes to synaptic dysfunction following challenge with $A\beta_{25\text{-}35}$.

The sex specific behavioral phenotypes observed by Chevallier *et al*. (2011) leads to the hypothesis that brain wide loss of sig-1R may be influencing female and male brains differently. While investigating these differences would likely provide interesting results, it is beyond the scope of this thesis. Therefore, I focus on male mice only. It is critical to consider male and female mice separately, not only because of their different behavioral phenotype, but also because of a known interaction between sig-1R and female sex hormones, such as estradiol and progesterone sulfate (Chevallier *et al*., 2011). Male mice were selected for the initial investigation to avoid these potentially confounding variables.

These experiments were designed to examine several aspects of cellular physiology. First, I examined characteristics of AP firing to determine if loss of sig-1R changes both the number of APs fired as well as their properties. I next looked at whether there are changes to presynaptic function by recording PPR, which looks at the probability of presynaptic vesicle release, and mEPSCs, which looks at the AMPA component of the synapse. Finally, I looked for changes in post-synaptic receptors, namely the AMPA/NMDA ratio to determine changes in receptor populations and NMDAR decay kinetics for an indication of NMDAR subtype populations. These basic qualities of synaptic physiology will provide some insight into any potential differences between the two genotypes. The results of these experiments are also important for future plans to explore the role of the sig-1R in an animal model of AD, which will be discussed further in chapter 4.

4.2 Results

4.2.1 Action Potential Firing Characteristics

The ability of neurons to fire APs is critical for signals to be passed through the brain and body. My goal was to determine if loss of sig-1R induces changes to cell and synaptic physiology. I first examined basic AP firing characteristics of the WT and KO mice in CA1 hippocampal neurons in current clamp mode using 1-second voltage steps, from -40 pA to +360 pA. **Figure 12 A & B** show representative traces of AP firing following a 300pA step current. **Figure 12C** shows a plot of action potential spike count versus current injection. While it appears that Sig-1R KO mice fired fewer APs for a given current injection, no significant differences were found (Two-way Repeated measures ANOVA, p=0.6697). At the maximum injected current WT (n=6) mice fired an average of 34.33 APs compared to the KO mouse count

(n=7) 27.7 (*t*-test, p=0.1049). At the end of each current step an afterhyperpolarization (AHP) is observed (**Figure 12 A, B**). The size of this post-train AHP can influence firing rate of a neuron as well as be used as a proxy of calcium dynamics within the cell. This latter point is due to the fact that calcium accumulates within the cell when each AP is fired. K^+ channels, some of which are Ca^{2+} activated, mediate the AHP. Therefore changes in the amplitude of the AHP can indicate that more (or less) calcium is being released into the cell. **Figure 12D** shows a plot of AHP amplitude versus current injection. Analysis of the AHP showed no significant changes at the maximum current injection (p=0.51). Two-way repeated measures ANOVA analysis was also not significant (p=0.3684). These experiments also allowed analysis of other cellular and synaptic characteristics summarized in **Table 3**. No significant differences were found when comparing the resting membrane potential, threshold to fire an AP, AP peak amplitude, AP half width and the 20-80% AP rise time between sig-1R KO and WT mice (**Table 3**). The overall profile of action potentials in the KO was not significantly different than the WT mice but did show a weak trend of reduced action potential firing.

4.2.2 mEPSC

The miniature EPSC (mEPSC) shows the spontaneous occurrence of vesicle release that is not a result of presynaptic depolarization. To prevent presynaptic cell depolarization Na^+ channels were blocked by TTX (500 nM), $GABA_A$ receptors were blocked by picrotoxin (100 μM) and glycine receptors were blocked by strychnine (500 nM) This parameter is indicative of pre and post synaptic qualities of AMPA containing synapses (frequency of events) as well as the post-synaptic strength of the AMPARs (amplitude of the event**).** . **Figure 13A&B** show representative traces of mEPSC recorded in cells from WT and sig-1R KO mice. Multiple

analyses performed on the recordings include the frequency, inter-event-interval, amplitude and current decay. The frequency of the mini events in the WT mice was 0.97 +/- 0.25 Hz and 0.83 +/- 0.17 Hz in the KO mice (p=0.658) (**Figure 13C**). The average inter-event-interval for the WT and KO were 0.991 +/- 0.146 seconds and 1.321 +/- 0.219 seconds, respectively (p=0.2440) (**Figure 13E**). Average event amplitude was 13.95 +/- 1.27 pA in the WT mice and 12.61 +/- 1.24 pA in the KO (p=0.47) (**Figure 13D**). Event decay is analyzed to see if there are changes within the population of AMPARs present at the synapse. The decay of the events was 17.86 +/- 2.91 milliseconds and 12.59 +/- 1.44 milliseconds for the WT and KO mice, respectively, and did not show statistical significance (p=0.1568) (**Figure 13F**). These results suggest that there is a similar population of AMPA containing synapses in the WT and knockout mice.

4.2.3 Paired Pulse

To determine changes in the probability of release of glutamate vesicles from presynaptic terminals I analyzed the paired pulse ratio (PPR). The PPR (EPSC2/EPSC1) is determined by the ratio of the second evoked EPSC (EPSC2) to the first evoked EPSC (EPSC1) at a set interval (**see Figure 14 A & B**). For this experiment an interstimulus interval of 100 milliseconds was used at a frequency of 0.067 Hz. These results can be interpreted as higher values of PPR, showing paired pulse facilitation, indicate a low probability of release and lower PPR values, showing paired pulse depression, indicate a high probability of release. It is well established in the literature that the CA3-CA1 synapse typically shows paired pulse facilitation. In fact this is what I observed. **Figure 14A&B** shows representative traces of EPSCs evoked by stimulation of the Schaeffer collateral pathway for both WT and KO animals. In the case of the WT and KO mice, no significant difference (p=0.1586) was found between the paired pulse ratios of the WT

(1.78 +/- 0.15, n=4) versus the KO mice (1.42, +/- 0.16, n=6) (**Figure 14C**). Both genotypes showed paired pulse facilitation. These results suggest that both genotypes have similar presynaptic mechanisms of vesicle release.

4.2.4 AMPA/NMDA ratio

As mentioned previously, the AMPA and NMDA receptors are the two main post-synaptic receptors that respond to the release of glutamate. I investigated the AMPA/NMDA ratio to determine if there are changes in the population of glutamatergic ion channels. Alterations in this measure can give some indication of changes in synaptic strength. A change in the ratio can indicate a change in the function or amount of AMPARs, NMDARs or both. Excitatory postsynaptic current (EPSC) were evoked by a stimulating electrode while the cell was voltage-clamped at +40mV. This elevated holding potential removes the Mg2+ block from the NMDAR and allows current to flow through the receptor. The mixed AMPA, NMDA EPSC was isolated in the presence of picrotoxin (100μM) (to block GABA receptors) and recorded. I first recorded this mixed current and then to isolate the AMPA component of the EPSC, D-APV (50μM) was applied to block the NMDA component. Post analysis subtraction of the isolated AMPA component from the mixed current gave the NMDA EPSC. Figure **15 A & B** show representative traces of the mixed current in red, the AMPAR component in blue, and the resultant NMDAR component in pink from WT and KO animals. The results of this experiment (**Figure 15C**) show that the AMPA/NMDA ratio of the WT mice (0.42 +/- 0.14, n=5) is not significantly different (p=0.7872) than that of the KO mice (0.47, +/- 0.09, n=8). Thus, we have no evidence to suggest that loss of sig-1R changes the synaptic strength and weight of CA1 hippocampal neurons compared to WT.

4.2.5 NMDAR Kinetics

As discussed, the GluN2 subtype of NMDAR greatly affects the kinetics of the channel. This has important consequences for the cell. We therefore wanted to determine if the NMDAR composition was similar between WT and KO animals. The NMDAR current was isolated by voltage clamping the neuron at -30mV in the presence of picrotoxin and NBQX. The bi-exponential decay of the NMDAR was analyzed offline. The weighted tau (τ_{mean} = $[A_f/(A_f+A_s)]\tau_f + [A_s(A_s+A_f)]\tau_s$) which accounts for the fast and slow components of the NDMAR decay was determined in post analysis (Stocca & Vicini, 1998). **Figure 16A&B** show representative traces of the NMDAR current. The decay refers to the period immediately after the peak of the current to when the recording returns to the baseline. The calculated weighted tau of the WT and KO mice were 78.1139 +/- 3.663 milliseconds and 69.0519 +/- 3.634 milliseconds , respectively, and were found to not be significantly different (p=0.1296) (**Figure 16C**). These results suggest that there are comparable relative proportions of NMDAR subtypes at the synapses of these mice.

4.3 Discussion

This experimental series investigated the basic electrophysiological properties of CA1 pyramidal neurons of KO and WT sig-1R mice. In these basal conditions, no significant differences were found. These results are not particularly surprising given that I only focused on male mice that have been reported to show an anxiety behavioral phenotype. It is more likely that alterations in the ventral hippocampus or amygdala would contribute to an anxiety phenotype (Roozendaal *et al*., 2009). My focus was on the dorsal hippocampus because it is a

very important region of the brain in regards to learning and memory and it is one of the first brain regions to accumulate Aβ plaques in AD. The results I generated are an important foundation for further investigations into the role of the sig-1R and synaptic physiology in AD.

The AP firing results show that the sig-1R is not essential for generating and propagating trains of action potential in the CA1 pyramidal neurons of these mice. This result is a bit surprising given that the sig-1R is known to interact with both Na^+ and K^+ channels, the two receptors responsible for AP generation (Johnston *et al*., 2003). As previously discussed, the activation of the sig-1R by an agonist has been shown to inhibit Na^+ and K^+ channels. In one sense inhibiting Na^+ channel opening could decrease cell excitability and reduce APs. On the other hand, inhibiting the K^+ from opening could make the cell more excitable. What is more likely is that the sig-1R is not constitutively acting on these channels. It is hard to say if this is occurring in the sig-1R KO mice but either way they are able to exhibit sustained AP trains. APs are obviously essential for propagating signals within and between brain regions and from an electrophysiological perspective this ability is fully intact in the CA1 pyramidal neurons of these KO mice.

Looking specifically at the synaptic physiology of the KO mice, similar values between the two genotypes were found. A similar AMPA/NMDA ratio indicates that there are a comparable proportion of these two glutamatergic receptors at these synapses. I next looked at mEPSCs to further dissect the AMPA component of these synapses. I found similar values of frequency, amplitude, inter-event-interval and decay kinetics. The frequency and inter-event-interval suggest that there is a similar probability of spontaneous, non-depolarization dependent, vesicle release at AMPAR containing synapses. The amplitude and decay kinetics show that the strength of AMPARs at each synapse is similar. These results were not entirely expected because

it has been shown that activation of the sig-1R caused an increase in neurite out growth (Kimura *et al*., 2013). If the sig-1R was a necessary component of neuron growth I could possible expect there to be fewer synapses and therefore fewer mEPSC events in the KO mice, but that was not observed.

The PPR values were also similar between genotypes. Differences in PPR would indicate a change in the presynaptic functioning and probability of release of the synapses in question, in this case it is the Schaffer collaterals pathway of CA3 pyramidal neurons. The release of neurotransmitter vesicles at the synapse is triggered by the influx of calcium caused by the depolarization of the presynaptic terminal. It was conceivable that there may be changes in PPR in the KO mice because of the well-documented interactions between sig-1Rs and VGCCs (Mueller *et al*., 2013). The sig-1R also has a role in intracellular Ca^{2+} dynamics (Su *et al*., 2010). However, despite these indications, the synaptic vesicle release machinery of the KO mice is intact.

The lack of changes in the sig-1R KO mouse could be due to compensatory mechanisms that occur early in the development of these mice. As I mentioned in the introduction to this chapter, ligand-binding assays showed that the sig-2R is likely present in these mice. The sig-2R and its functional implications within neurons are largely unknown but it may be the explanation for the lack of difference in my results. As the sig-1 KO mouse is a complete KO, compensatory mechanisms may happen early in development. Conversely, it has been suggested in the literature that the sig-1R is constitutively inactive under normal, healthy conditions and only becomes important during states of cellular stress (Kourrich *et al*., 2012). Further investigation is definitely warranted.

Given the discrepancy between sig-1R KO male and female mice, a future goal will be to look at the same electrophysiological characteristics in female mice. It is reasonable to hypothesize that because the hippocampus has been implicated in spatial memory performance, the female mice could show changes in their CA1 pyramidal neuron physiology. Another important consideration to address in the characterization of the female mice is what stage of the estrous cycle they are in because of the link between sig-1R and female sex hormones such as estradiol. Ovariectomizing (ovx) the female mice is a consideration because it will eliminate the influence ovarian steroids. Chevallier *et al* (2011) were able to rescue the spatial memory deficits in the female KO mice by treating them with estradiol. By using ovx mice in the electrophysiological characterization it could also enhance the phenotype and provide a clearer picture of the consequences of sig-1R KO.

This initial characterization of the sig-1R KO mouse is a stepping-stone to further experimentation looking at the role of sig-1R in synaptic physiology. Understanding the basal conditions of these mice is necessary before going further. Our lab is deeply interested in the pathophysiology of AD. The goal of understanding how the sig-1R is implicated in AD is the focus of chapter 5.

Chapter 5: Aβ Challenge of Sig-1R Knockout Mice

5.1 Introduction

As previously mentioned, AD is a debilitating disease for which there is no cure and only minimally effective therapies. Understanding the underlying pathophysiology is crucial to finding new pharmacotherapeutics. Our lab has a long standing interest in the sig-1R and there is evidence that it could play an important role in AD. The sig-1R has been implicated in certain populations of Alzheimer's patients through polymorphisms in the *SIGMAR1* gene and the association of these polymorphisms with the AD risk factor APOE4 (Maruszak *et al*., 2007; Feher *et al*., 2012). A *SIGMAR1* gene variant, TT-241-240P2, in Japanese populations, has been shown to decrease sig-1R expression and be associated with a reduce susceptibility to AD (Uchida *et al*., 2005). Conversely, Mishina *et al*. (2008) found that AD patients had decrease sig-1R binding sites in the CNS. Additionally, strong evidence for the role of sig-1R in AD from a pharmacological perspective comes from the commonly prescribed acetylcholinesterase inhibitor donepezil sold as the trade name Aricept. Donepezil has EC50 value of 14.6 nM for the sig-1R (Kato *et al*., 1999). When used to treat mice that received a single intracerebroventricular (i.c.v.) injection of $A\beta_{25\text{-}35}$, donepezil showed neuroprotective and anti-amnesic properties that were attenuated by the sig-1R antagonist BD1047 (Meunier *et al*., 2006).

In animal models, the sig-1R ligand DTG and DHEA were able to prevent $A\beta_{25\text{-}35}$ induced memory impairments (Maurice & Lockhart, 1997, Maurice, Su & Privart, 1998). Sig-1R ligands have also been demonstrated to rescue spatial memory deficits in aged mice (Phan *et al*., 2003). In cultured cortical neurons, cells challenged with $A\beta_{25\text{-}35}$ showed less cell death when

treated with sig-1R agonists, further strengthening the theory that sig-1R is neuroprotective (Marrazzo *et al*., 2005).

There is a growing body of knowledge pointing to calcium dysregulation being an underlying component of AD, which tracks well with the potential implications for the role of sig-1R in AD. Stutzmann *et al*. (2004) showed that IP3Rs function was enhanced in the transgenic mouse for the AD linked gene *presenilin 1*. The same group also demonstrated that calcium release from the ryanodine receptor was greater in 3xTg AD mouse CA1 pyramidal neurons (Chakroborty et al., 2009).

Looking at the MAM in an amyloid precursor protein $(APP)_{Swe/Lon}$ mouse model of AD and samples of human brain with AD, Hedskog et al. (2013) revealed that the sig-1R was necessary for neuronal survival and was up regulated in the mouse model. Knocking down sig-1R with siRNA in mouse model primary hippocampal cultures resulted in neuronal death. Treating the mouse model with Aβ also increased the contact points between the ER and mitochondria offering further evidence for its importance in the pathology of AD.

Sigma-1 receptors have been demonstrated to interact with and modulate several voltage-gated and ligand gated ions channels that shape action potential firing characteristics as well as intracellular ion channels responsible for calcium transport and regulation. Given that the sig-1R has been repeatedly shown to have neuroprotective effects and implicated to play a role in the neurodegenerative progression of AD the goal is to determine what deficits or changes occur when the knockout mice are challenged with an $A\beta_{25-35}$ assault. Initially, the AP firing and AHP of $A\beta_{25-35}$ infused WT and KO mice will be investigated to determine if basic changes to these characteristics occurs. We are particularly interested in the AHP in the $A\beta_{25-35}$ infused sig-1R

KO mice because if there is a dysregulation in Ca^{2+} dynamics it will be a good initial screen for such activity.

5.2 Results

Similar to chapter 3, I investigated the AP and AHP characteristics of WT and KO mice that received a single intracerebral ventricular injection of $A\beta_{25-35}$ two weeks prior to recording. Preliminary results that I have obtained so far are from $A\beta_{25-35}$ treated WT and KO mice and reverse treated WT mice. Future experiments are aimed to add in KO mice treated with the vehicle and increase the n value. However, given the results from chapter 3, we do not anticipate seeing a difference between these WT and KO vehicle treated animals provided that the injection itself does not manifest a significant detriment to the mice. Following a similar protocol as before I patched CA1 pyramidal neurons in current clamp mode but in this experiment took them through a series of 10 mV steps from -20 to +250mV. The smaller step provides more data points for steps that cause fewer APs to fire. The analysis of my data looked at three different aspects of the data recorded. First, I looked at the number of action potentials fired in relation to the amount of current injected. It is notable that there are fewer APs fired in the $A\beta_{25-35}$ treated KO animals than WT $A\beta_{25-35}$ and reverse treated WT animals (**Figure 17A-C**). The appearance of the action potential trains is also noticeably different in the treated KO cells (**Figure 17C**). **Figure 17D** compares the AP spike count to current injected and finds there is a significant interaction between the groups by two-way repeated measures ANOVA ($p=0.0212$). The next parameter analyzed looks at the post train AHP versus current injection. In this analysis the WT and KO $A\beta_{25-35}$ treated cells appear to exhibit a greater AHP than the vehicle treated WT animal (**Figure 17E**). Two-way repeated measures ANOVA finds the interaction between the groups

significant as well ($p<0.0001$). The AHP is mediated by the outward flow of K^+ as a result of the cell being depolarized by APs, therefore the number of APs can influence the size of the AHP, in part due to greater [Ca^{2+}] gating K^+ channels. To investigate this, the AP count was compared to the magnitude of the AHP. This analysis shows that there is a greater AHP for fewer APs in the $A\beta_{25-35}$ treated KO mice as compared to treated and untreated wild type mice (**Figure 17F**). Data at this point is still preliminary which prevents statistical analysis. The larger AHP with fewer APs in the $A\beta_{25-35}$ treated sig-1R KO mice is somewhat paradoxical. One explanation could be that there is a greater amount of Ca^{2+} influencing the AHP *via* the SK channels or there many be changes in the VGCC, however, the source of this Ca^{2+} would not appear to be due to depolarization, this finding is of particular interest to us and definitely warrants further investigation.

5.3 Discussion

The preliminary results of the AP and AHP characteristics of the $A\beta_{25-35}$ treated sig-1R KO mice presents some insights into the consequences of sig-1R loss and interesting prospects for future studies. The change in the AHP and accommodation in AP firing is of particular interest because it suggests a change in the calcium dynamics of the cell. In AD, Aβ plaque accumulation is known to have several detrimental consequences for cells near by causing the accumulation of reactive oxygen species that destabilize the plasma membrane's functional abilities and cause the cell the be more excitable, in part due to greater intracellular [Ca^{2+}] (Kapogiannis & Mattson, 2011). In this diseased state the neurons have a greater influx of Ca^{2+} and dysregulation, which puts the neuron at greater risk of excitotoxicity and other detrimental effects. In regards to the AHP, higher [Ca^{2+}] could cause more Ca^{2+} gated K^+ channels to open

and drive the AHP. The sig-1R has been shown to be sensitive to ER Ca^{2+} stores and regulates the functioning of IP3Rs and ryanodine receptors (Hideaki et al., 2013). If the sig-1R could be shown to be important for regulating Ca^{2+} homeostasis in this animal model of AD it may offer new insights into what could be done to counter act the unstoppable progression of AD. It is also important to point out that the WT and KO $A\beta_{25-35}$ treated mice both exhibit a greater AHP yet the WT mouse is able to have more sustained AP trains than the sig-1R KO. Perhaps this data indicates a better management of intracellular Ca^{2+} when the sig-1R is present.

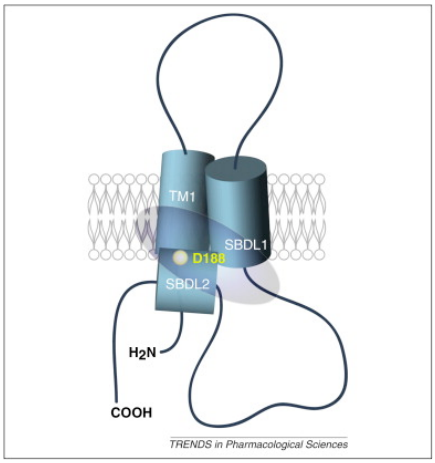
The next step in this animal model will be focused on determining if the changes seen in these mice are due to changes in Ca^{2+} homeostasis and if it is mediated by the ryanodine receptor and/or the IP3R. We feel this is the fist step to take given the role of the sig-1R at the MAM and its ability to modulate the potential receptors to blame for this perturbation in Ca^{2+}. To address this we will use a pharmacological approach to dissect which deviant receptor might be responsible. High concentrations of intracellular ryanodine or bath applied dantrolene block the ryanodine receptor (Isokawa & Alger, 2006). If this treatment is able to rescue the effect of $A\beta_{25-35}$ in the KO mice it would provide strong evidence for its role in the pathophysiology of AD.

Chapter 6: Conclusion

This investigation into the sig-1R and its implications for the pathophysiology of AD has revealed some interesting insights. In this thesis I explored the role of sig-1R and the modulation of NMDARs. Even though, I was unable to potentiate the NMDAR current in the heterologous expression model. I can say with some confidence that sig-1R probably does not modulate NMDAR by a direct mechanism but may use nearby signaling mechanisms. The characterization of the AP, AHP and synaptic physiology of CA1 pyramidal neurons in the sig-1R KO mouse demonstrates that cell signaling and communication mechanisms are intact in these animals. When the sig-1R mice were challenged with $A\beta_{25-35}$ some interesting differences did arise between the treated and untreated KO animals. The meaning that can be taken from this data is that, even though sig-1Rs are not essential for cell functioning under normal physiological conditions, in circumstances of cellular stress the sig-1R is likely critical for maintaining the some level of homeostasis within the cell. It has become more evident that the sig-1R is an important factor in the pathophysiology of AD. In fact, there are currently sig-1R agonists in clinical trials for AD. I look forward seeing where the work I have done in this thesis will take our understanding of the sig-1R in the pathophysiology of AD.

Chapter 7: Figures and Tables

A



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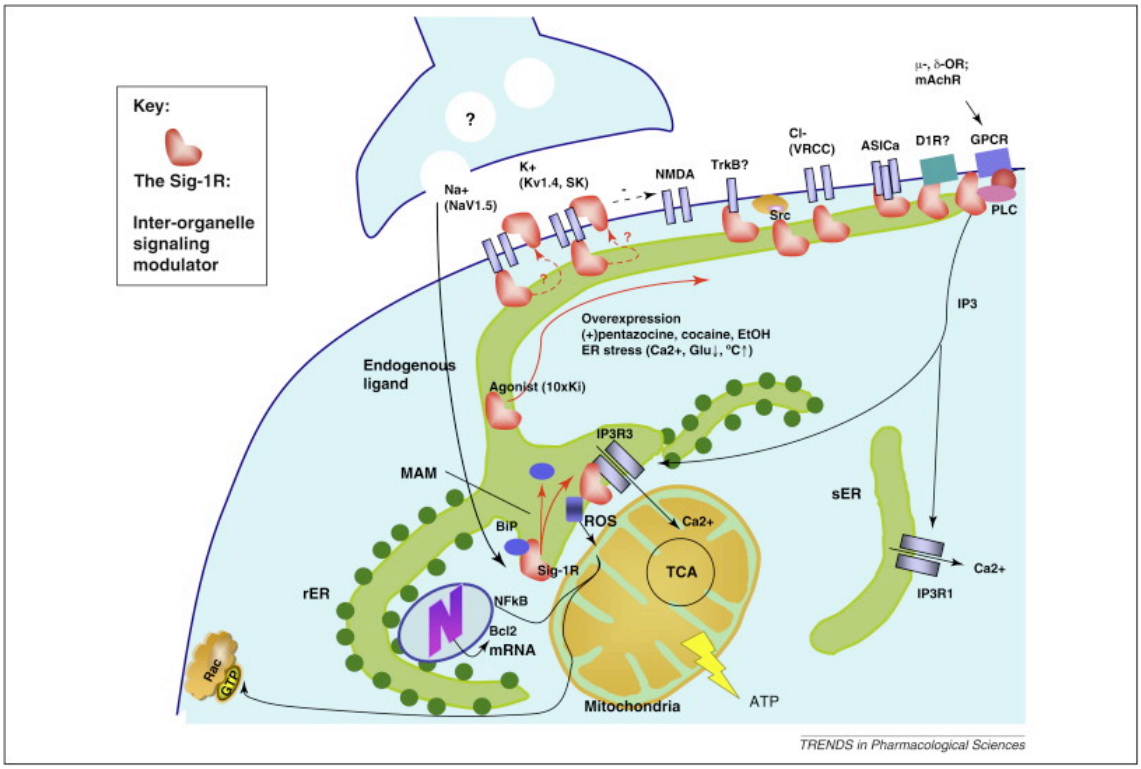

Figure 1. Proposed structure and known sig-1R interactions A) The proposed structure of the sig-1R is two transmembrane domains and a third hydrophobic region on the C-terminus tail, helping to from the shaded binding pocket B) Diagram of the known sig-1R interactions at the MAM and plasma membrane (Su *et al*., 2010)

Compounds	Subtype selectivity	Function
Benzomophans		
(+) SKF-10047*	sigma-1	agonist
(+) Pentazocine*	sigma-1	agonist
Dextromethorphane	sigma-1	agonist
(+)-3-PPP	sigma-1	agonist
Antipsychotic		
Haloperidol	sigma-1/2	antagonist
Spiperone	sigma-1/2	?
Pimozide	sigma-1/2	?
Antidepressant		
Imipramine	sigma-1	agonist
Fluoxetine	sigma-1	agonist
Fluvoxamine	sigma-1	agonist
Neurosteroid		
Progerterone	sigma-1	antagonist
Testosterone	sigma-1	?
DHEA-sulfate	sigma-1	agonist
Other synthetic compounds		
DTG	sigma-1/2	agonist
BD-1008	sigma-1/2	antagonist
PRE-084*	sigma-1	agonist
NE-100	sigma-1	antagonist
JO-1784	sigma-1	agonist
SA-4503	sigma-1	agonist
BD-1047	sigma-1	antagonist
BD-1063	sigma-1	antagonist

* drugs used in thesis (adapted from Hayashi & Su, 2005)

Table 1. The pharmacological profile of sigma ligands

Channel	Effect
Ca^{2+} channels	General inhibition (possible sig-1R drug crossreaction)
L-type	Potentiation (in CA1) (Sabeti *et al*., 2007)
L-type	Inhibition (retinal ganglion cells) (Tchedra *et al*., 2008)
K^+ channel	
Kv1.4	Inhibition (Zhang *et al*., 2010)
SK	Inhibition (Martina *et al*., 2007)
Na+ channels	
Nav1.2	Inhibition (Fontanilla *et al*., 2009)
Nav 1.5	Inhibition (Zhang *et al*., 2010)
I_{BK}	Inhibition (Zhang & Cuevas, 2005)
$I_{Na}{}^{a}$	Inhibition (Lupardus *et al*., 2000)
NMDAR	Potentiation (Martine *et al*., 2007)

Table 2. Plasma membrane ion channels and sig-1R activation effect

(adapted from Kourrich *et al*., 2012)

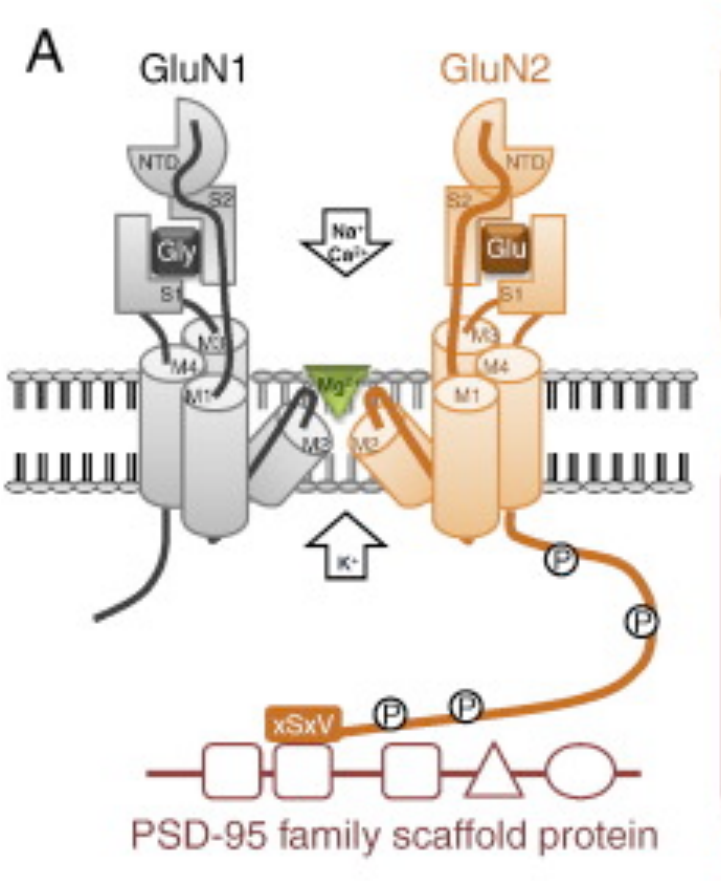

Figure 2. The structure of the NMDAR and PSD-95 A) Profile of a NMDAR showing the glycine binding site on GluN1 and glutamate binding site on GluN2. The channel formed by the tetramere in permeable to Na+, K+ and Ca2+. GluN2 subunit anchors to the PDZ domain of PSD-95. (Bard & Groc, 2011).

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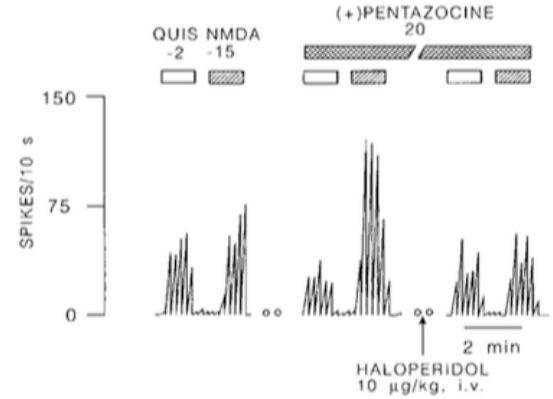

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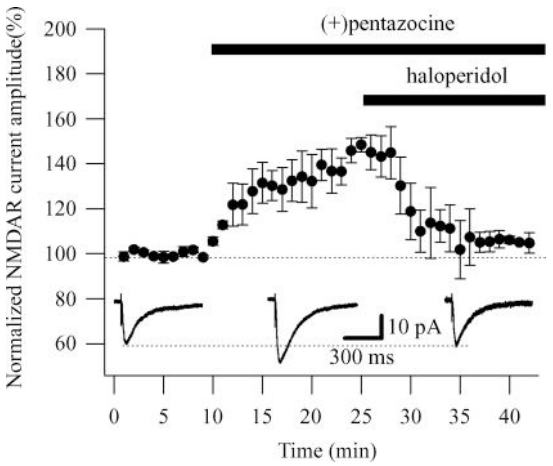

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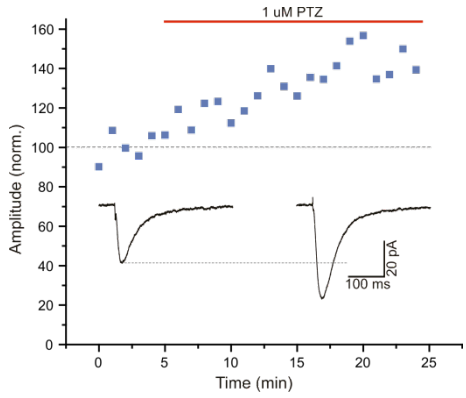

Figure 3. Functional effect of sig-1R ligands on NMDAR A) Potentiation of integrated firing rate histogram in CA1 dorsal hippocampus specific to NMDAR (Debonnel *et al*., 1996) B) Potentiation of isolated NMDAR current in rat CA1 (Martina *et al*., 2007) C) Preliminary (n=2) potentiation of isolated NMDAR in mouse CA1

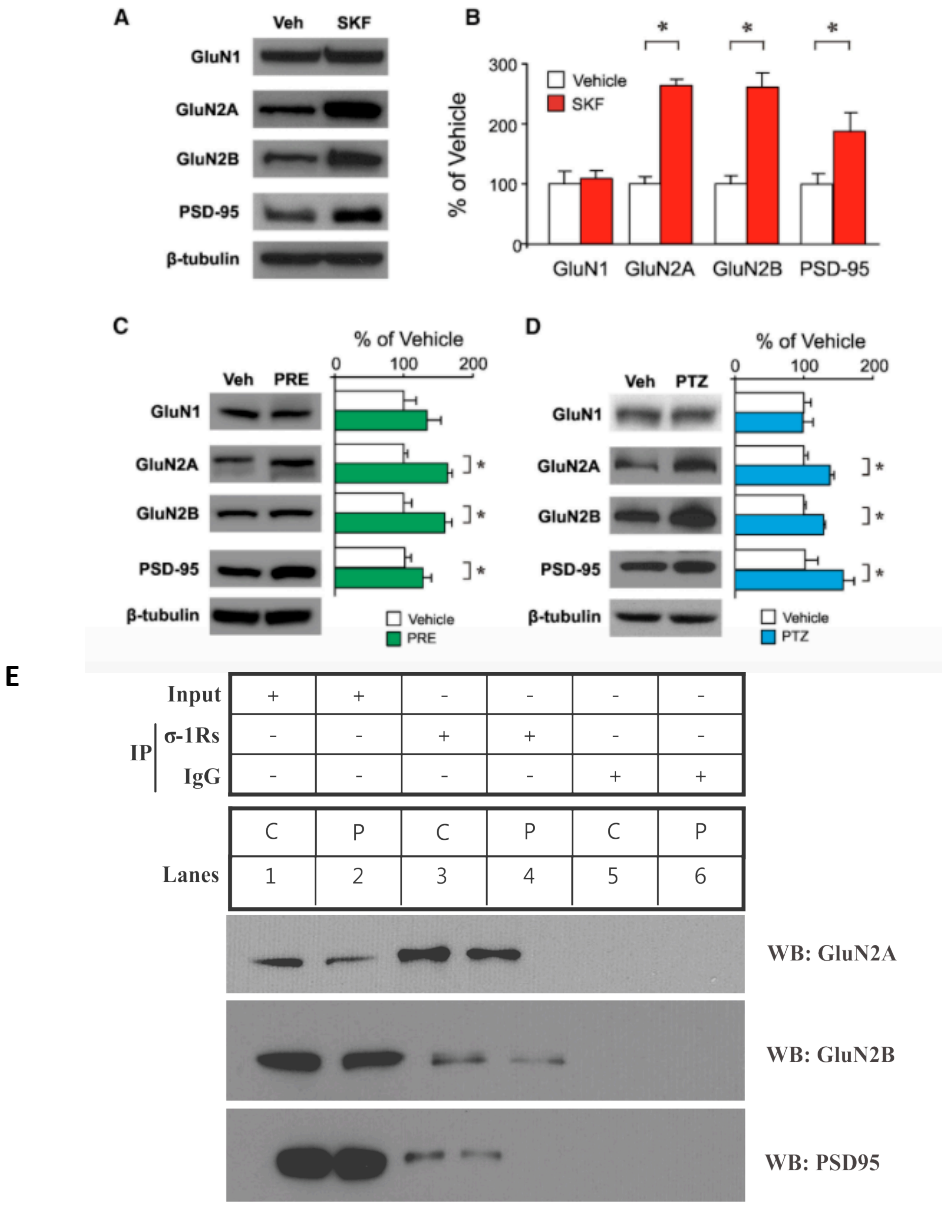

Figure 4. sig-1R ligands increase expression of NMDAR subunits and PSD-95 and sig-1R co-immunoprecipitation The intraperitoneal injection of sig-1R agonists SKF, PRE-084 and PTZ increases the expression levels of GluN2A, GluN2B and PSD-95 (A-D) in the synaptosomal fraction. sig-1R co-immunoprecipitates with GluN2A, GluN2B and PSD-95 (E), lanes 3 and 4 in control, C, and treated (PTZ), P, samples (Pabba *et al*., 2014).

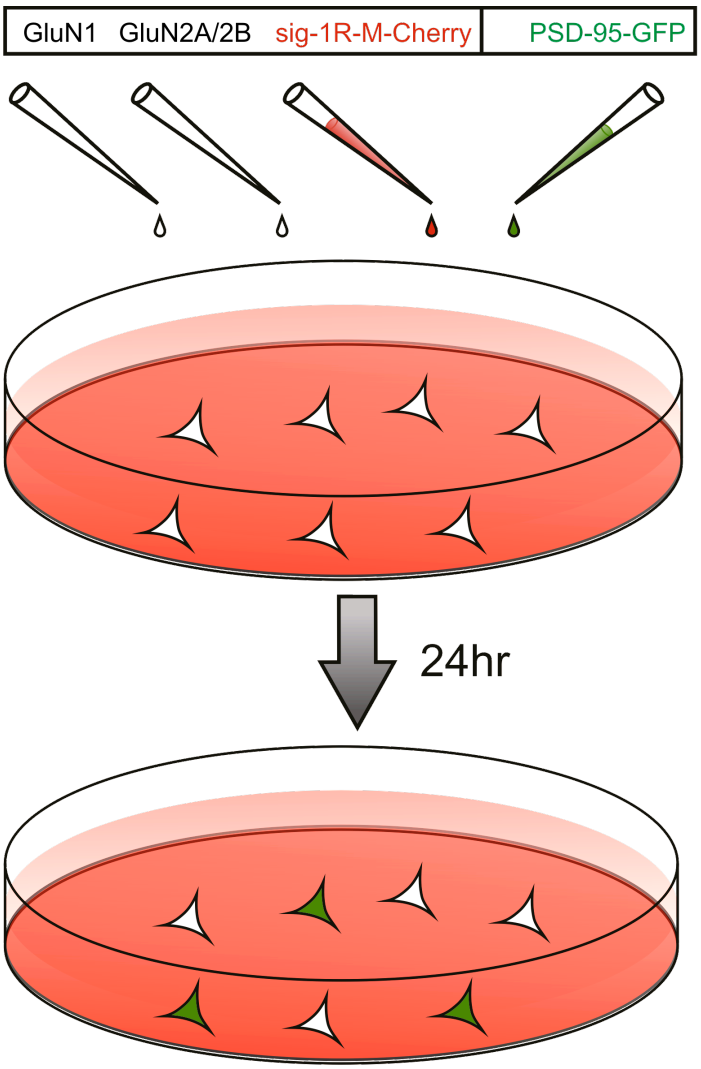

Figure 5. Transfection protocol HEK293 transfection paradigm of GluN1, GluN2AorB, sig-1R-m-Cherry and PSD-95-GFP. When transfecting without PSD-95, sig-1R-YFP and m-Cherry are used in place of sig-1R-m-Cherry and PSD-95-GFP.

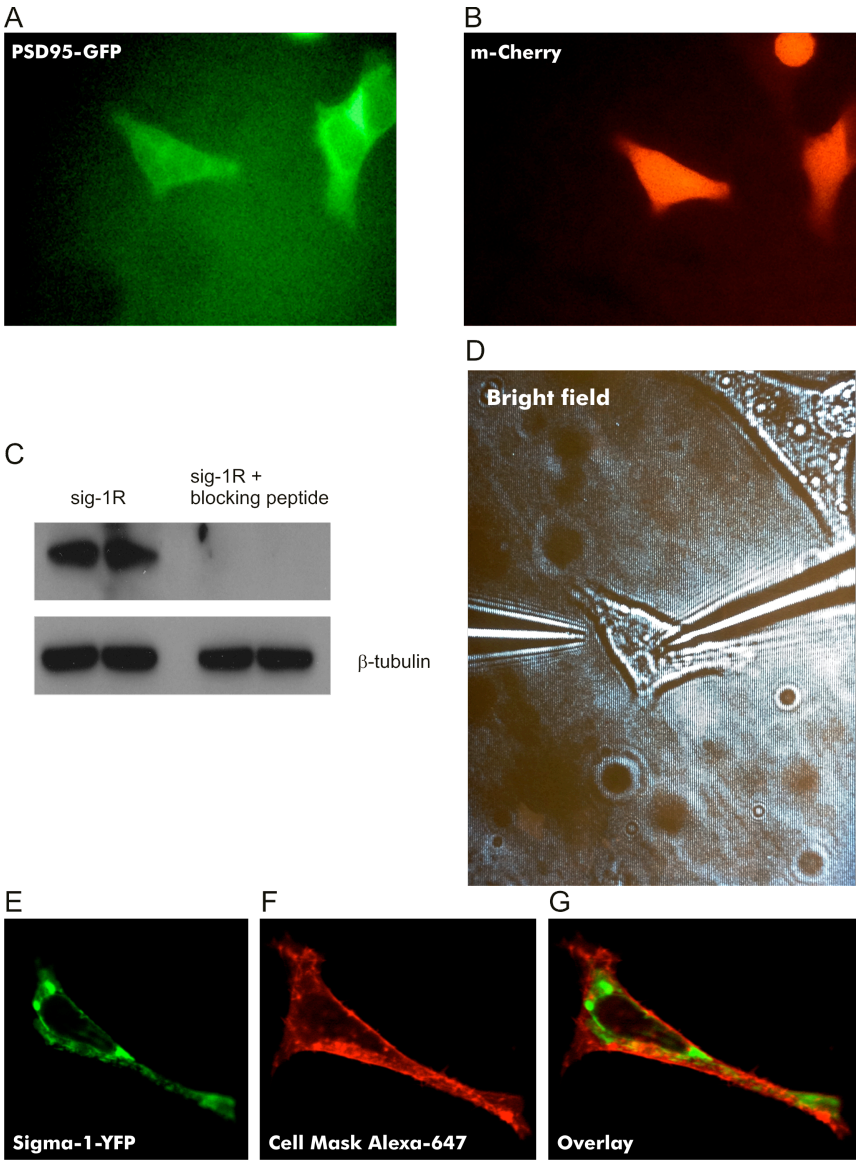

Figure 6. Transfected HEK293 cells and patching configuration Transfected HEK293 cells showing PSD-95-GFP (A) and m-Cherry (B). C) Western blot of endogenous sig-1R in HEK293 cells (Alex Sokolovski) D) Patching configuration of patching electrode on right and picospritzer on left. Confocal image of sig-1R-YFP (E), cell membrane marker Cell Max Alexa-647 (F) and overlay (G) (Adrian Wong).

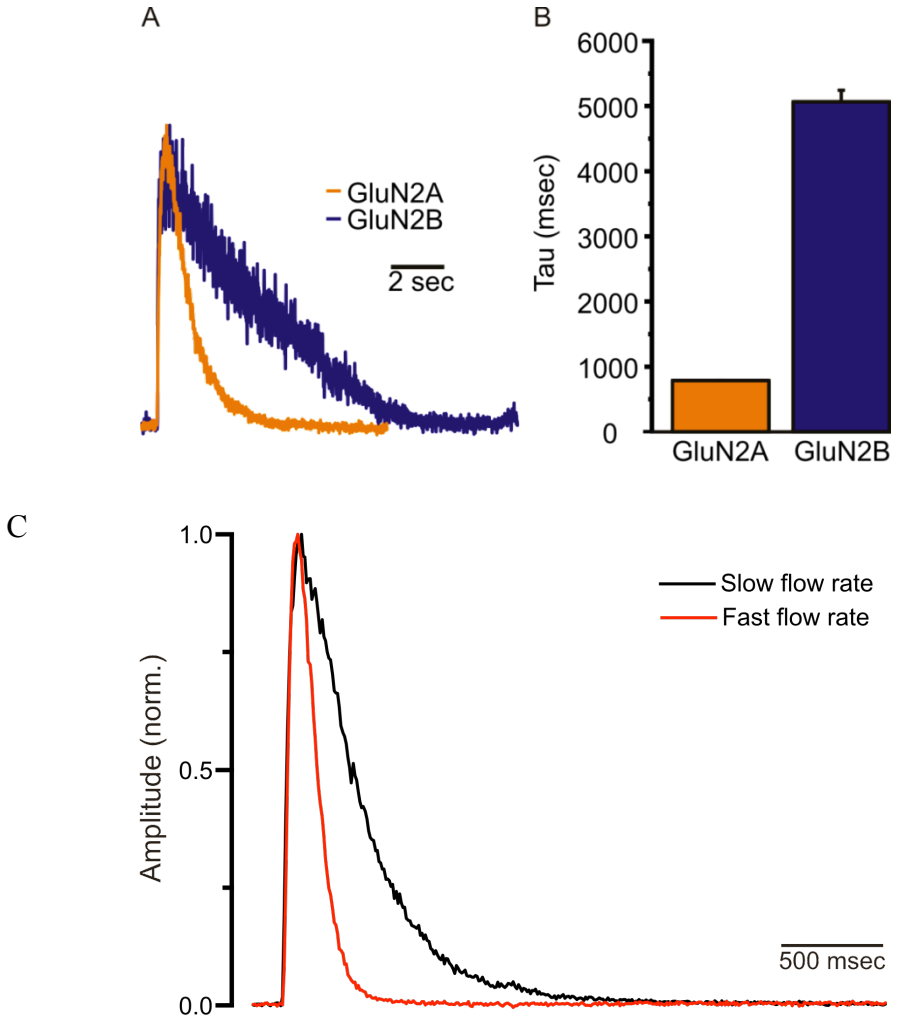

Figure 7. Decay kinetics of GluN2A and GluN2B NMDARS in HEK293 cells and flow change effect in GluN2A Normalized traces (A) of GluN2A and GluN2B NMDAR in HEK293 cells showing characteristic decay kinetics (GluN2B tau > GluN2B tau) (B). C) Demonstration of change in GluN2A NMDAR decay tau with changes in perfusion rate.

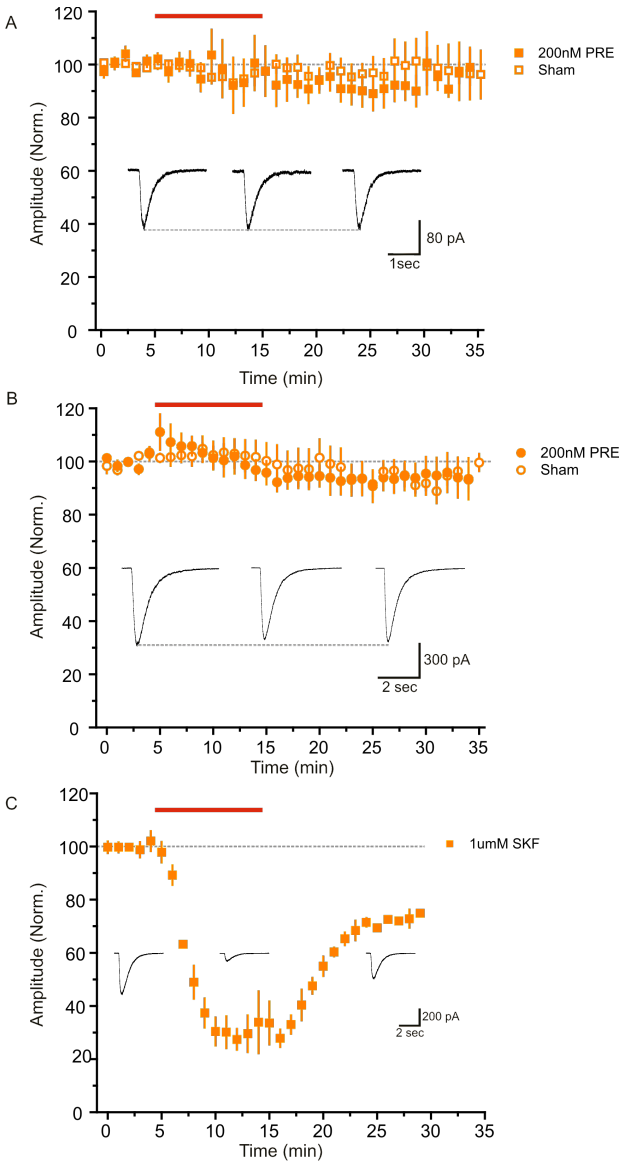

Figure 8. No potentiating effect of sig-1R activation on GluN2A NMDARs with or with out PSD-95 A) GluN2A NMDAR alone treated with 200 nM PRE-084 (p=0.6989, n=7) and Sham (p=0.8001, n=12) B) GluN2A NMDAR with PSD-95 treated with 200 nM PRE-084 (p=0.8017, n=5) and Sham (p=0.465, n=10) C) GluN2A NMDAR without PSD-95 treated with 1 μM SKF 10047 (p=0.0008, n=3). Red bar indicates 10 minute treatment period. Insets are representative traces of drug treated cells. Data points are normalized current amplitude +/- SEM. Significance calculated with *t*-test between time points 5 and 15 min.

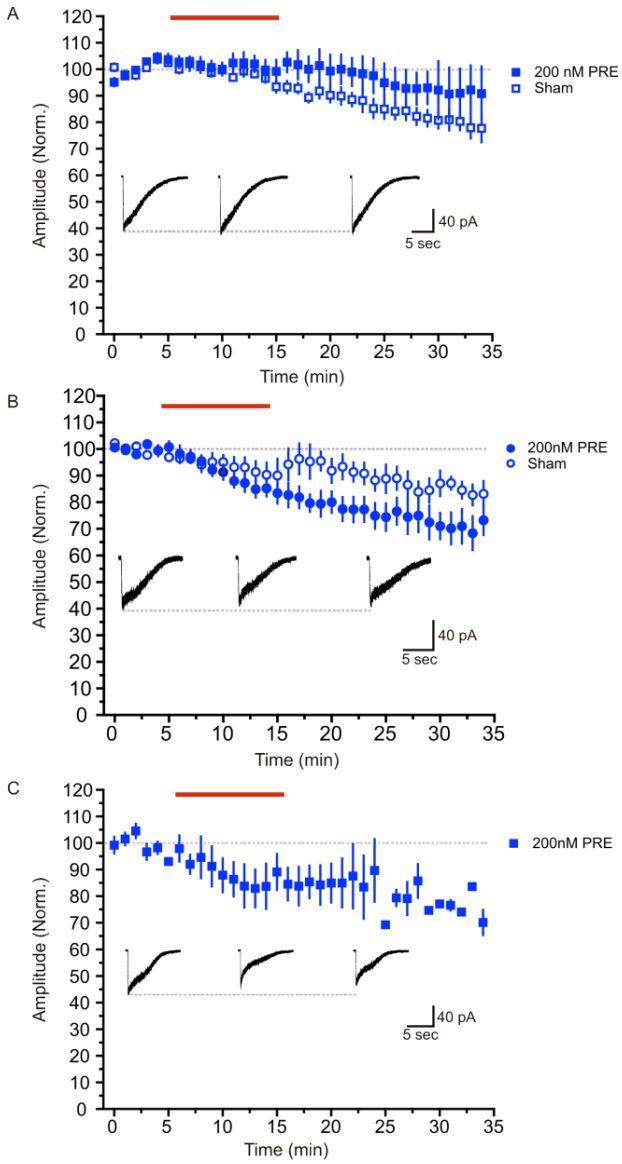

Figure 9. No potentiating effect of sig-1R activation on GluN2B NMDARs with or with out PSD-95 A) GluN2B NMDAR in the absence of PSD-95 treated with 200 nM PRE-084 (p=0.8017, n=8) and Sham (p=0.0776, n=8) B) GluN2B NMDAR with PSD-95 treated with 200 nM PRE-084 (p=0.0056, n=5) and Sham (p=0.1223, n=8) C) GluN2B NMDAR with PSD-95 treated with 200 nM PRE-084 with a K^+ Gluconate internal (p=0.1682, n=4). Red bar indicates 10 minute treatment period. Inset are representative traces of drug treated cells. Data points are normalized current amplitude +/- SEM. Significance calculated with *t*-test between time points 5 and 15 min.

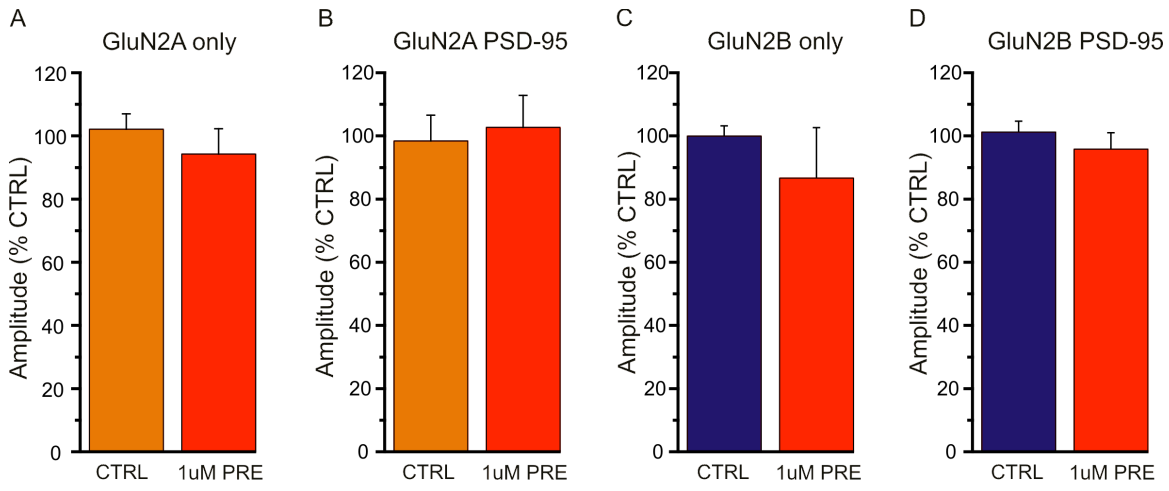

Figure 10. NMDARs do not potentiate with 1 μM PRE-084 treatment HEK293 cells transfected with GluN2A alone (A) (p=0.4168, n=7), GluN2A with PSD-95 (B) (p=0.7499, n=6), GluN2B alone (C) (p=0.2947, n=3) and GluN2B with PSD-95 (D) (p=0.9118, n= 2), treated with 1 μM PRE-084 for 10 minutes. Bars represent the control period (5 minute mark) and last minute of treatment period (15 minute mark). Error bars +SEM.

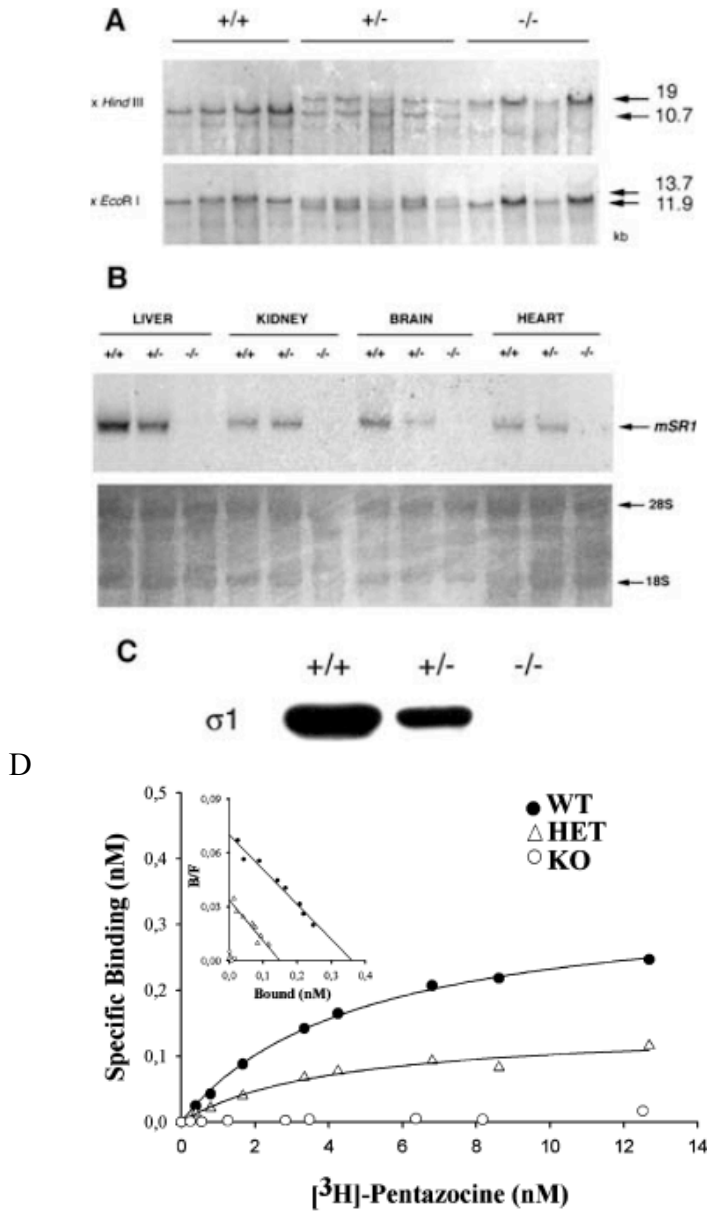

Figure 11. Confirmation of sig-1R KO The sig-1R knockout mouse was created with homologous recombination techniques and confirmed by Southern blot (A), Northern blot (B), Western blot (C) and ligand binding assay (D). (Langa *et al*., 2003)

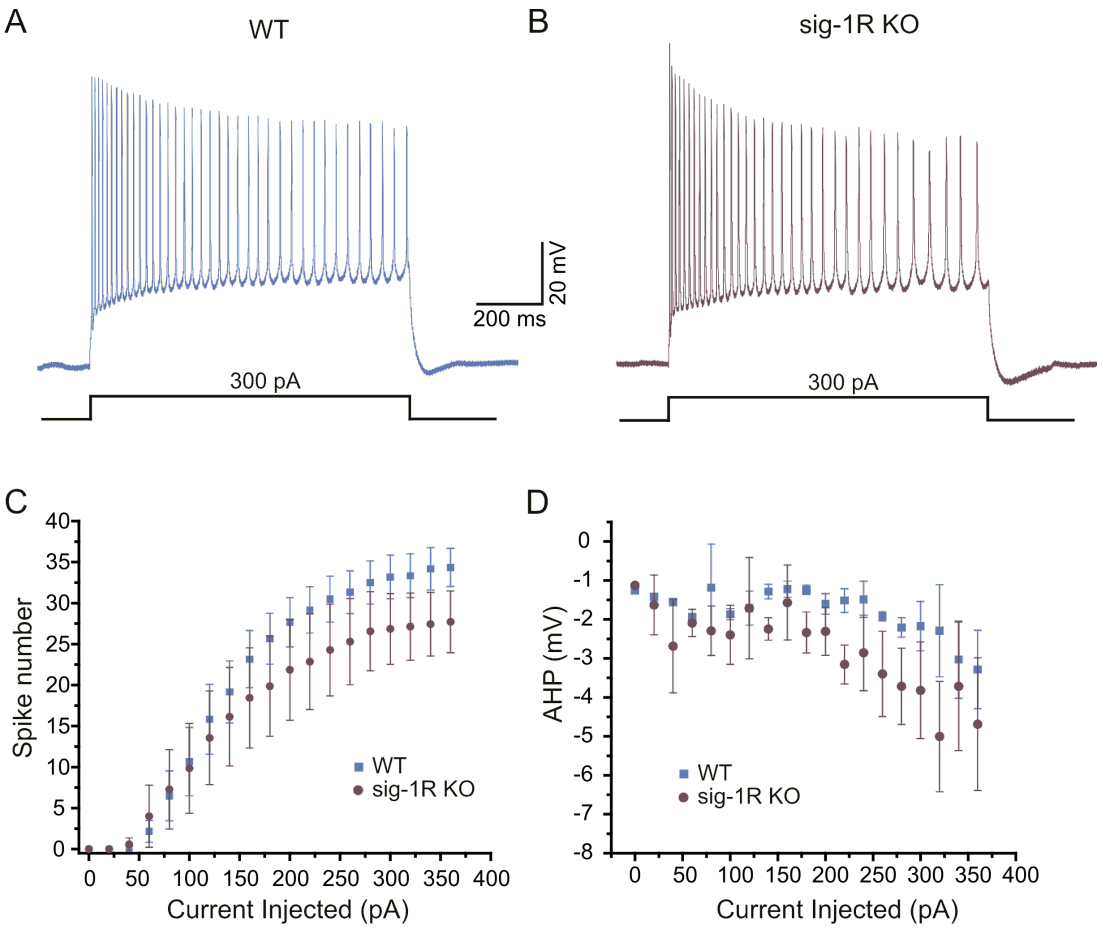

Figure 12. Action potential and after-hyperpolarization characteristics maintained in sig-1R KO mouse Representitive traces of WT (A) (n=6) and KO (B) (n=7) action potential traces of CA1 pyramidal neurons. Analysis of spike number versus current injection (C) (p= 0.1049 at 360 pA injection) Repeated Measures 2 way ANOVA not significant. AHP versus current injection (D) (p=0.5096 at 360 pA injection) Repeated Measures 2 way ANOVA not significant. Error bars +/- SEM.

	WT	KO
Resting Memb Pot (mV)	-67.7±1.1	-65.3±1.8
Threshold (mV)	-42.9±1.0	-41.1±1.2
Peak Amp (mV)	79.2±1.6	75.6±2.5
1/2 width (ms)	0.00097±4.2E-05	0.0011±7.3E-05
20-80% rise time	0.00023±9.5E-06	0.00027±2.6E-05

Table 3: Properties of action potential parameters in sig-1R KO mice

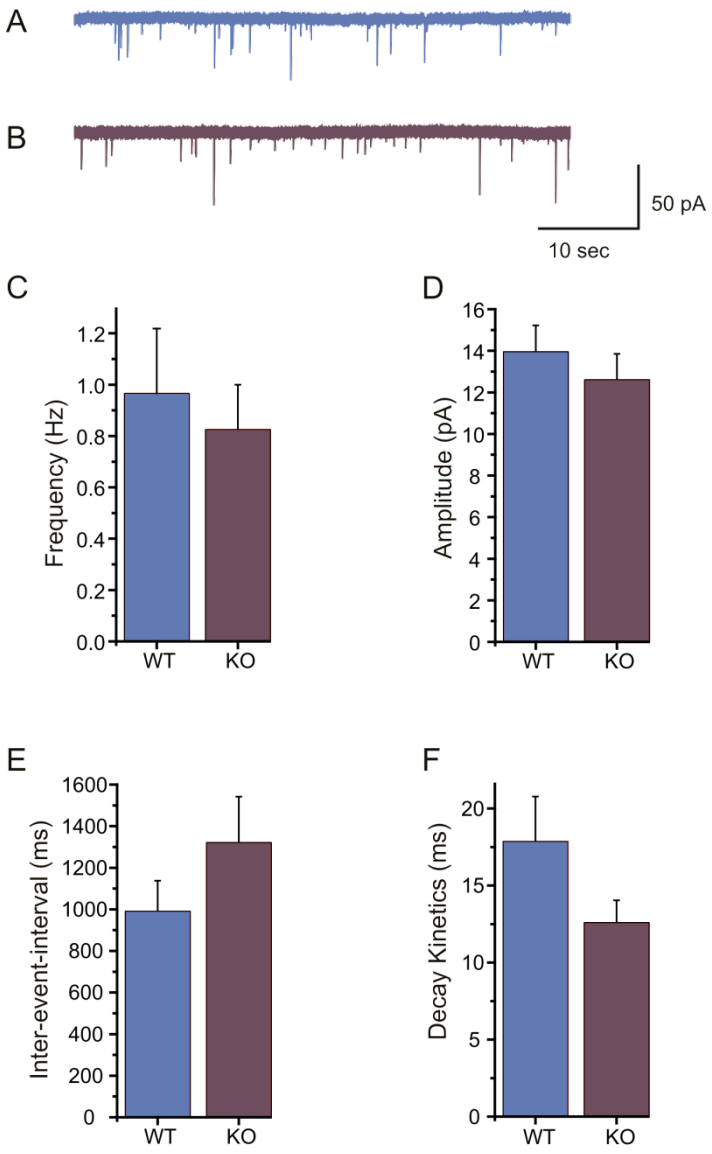

Figure 13. Miniature excitatory postsynaptic currents in sig-1R KO mouse are maintained mEPSC representative traces of WT (A) (n=5) and KO (B) (n=6) sig-1R mouse. Analysis of event frequency (C) (p=0.658), current amplitude (D) (p=0.4717), inter-event-interval (E) (p=0.2440) and AMPAR decay kinetics (F) (p=0.1568). Error bars +SEM.

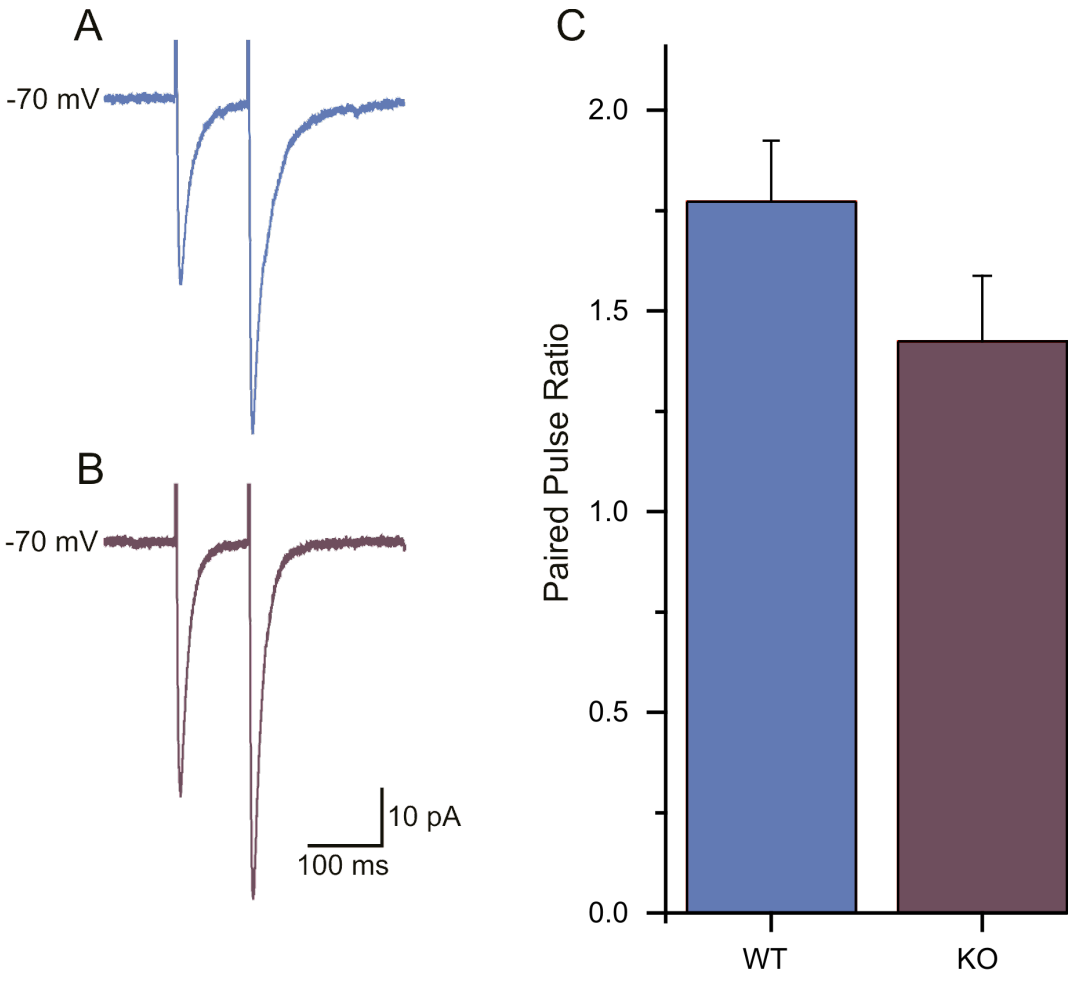

Figure 14. Probability of release is maintained in sig-1R KO mice demonstrated by paired pulse ratio of CA3-CA1 Schaffer collaterals synapse Representative traces of paired pulse recordings in WT mice (A) (n=4) and sig-1R KO (B) (n=6). Analysis of the ratio of the paired pulse ratio (C) (p=0.1586). Error bars +SEM.

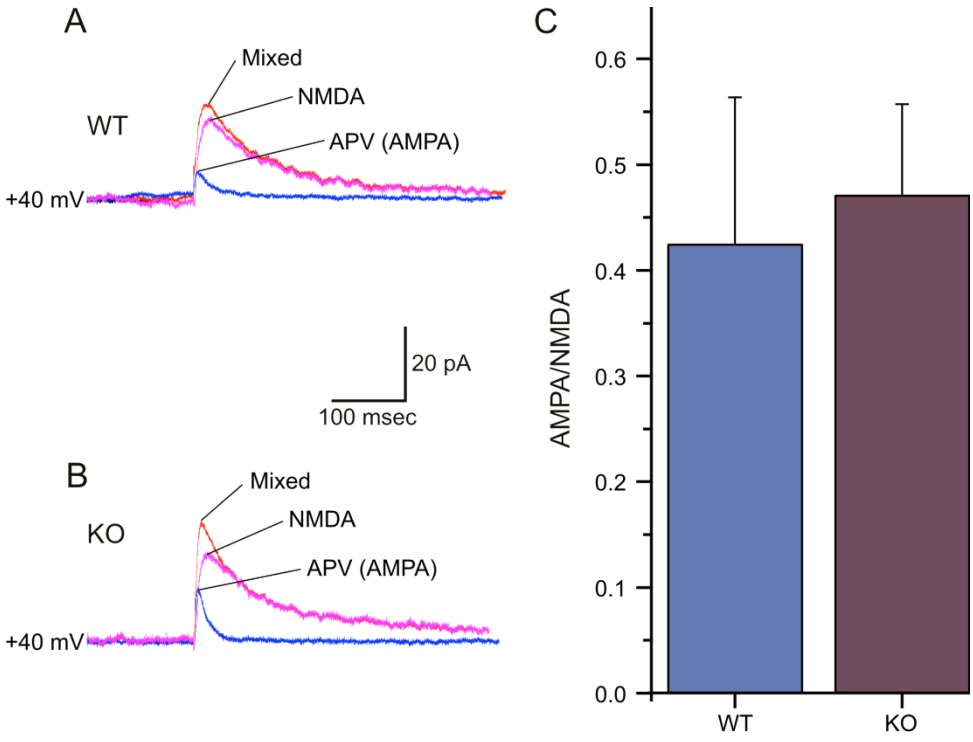

Figure 15. sig-1R KO mice have a similar relative proportion of AMPARs and NMDARs in CA1 pyramidal neurons Representative traces of +40 recordings from CA1 pyramidal neurons in WT mice (A) (n= 5) and sig-1R KO mice (B) (n=8). Red trace represents the mixed AMPA/NMDA current. Blue trace represents the pharmacologically insolated AMPA current. Pink trace represents the NMDA current derived offline by: Mixed current – AMPA current. Analysis of the AMPA:NMDA ratio (p=0.7872). Error bars +SEM.

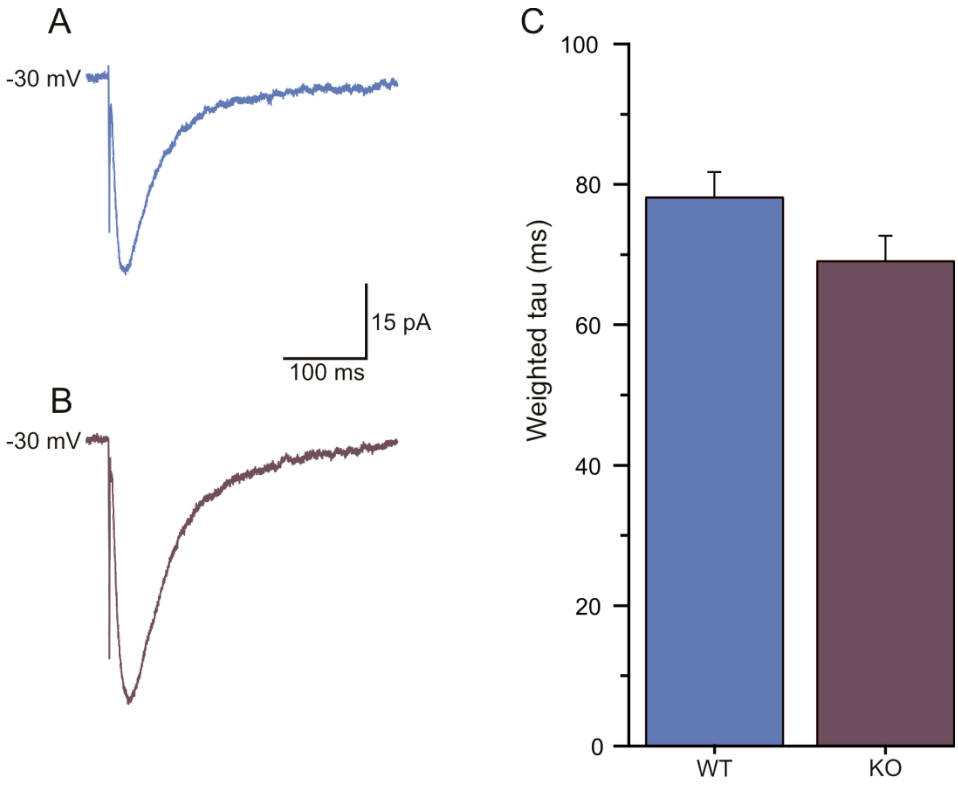

Figure 16. sig-1R KO mice have a similar profile of NMDAR subtypes in CA1 pyramidal neurons Representative traces of isolated NMDAR currents at -30 mV in WT (A) (n=4) and KO (B) (n=4) sig-1R mice. Analysis of NMDAR weighted decay tau (p=0.1296). Error bars +SEM.

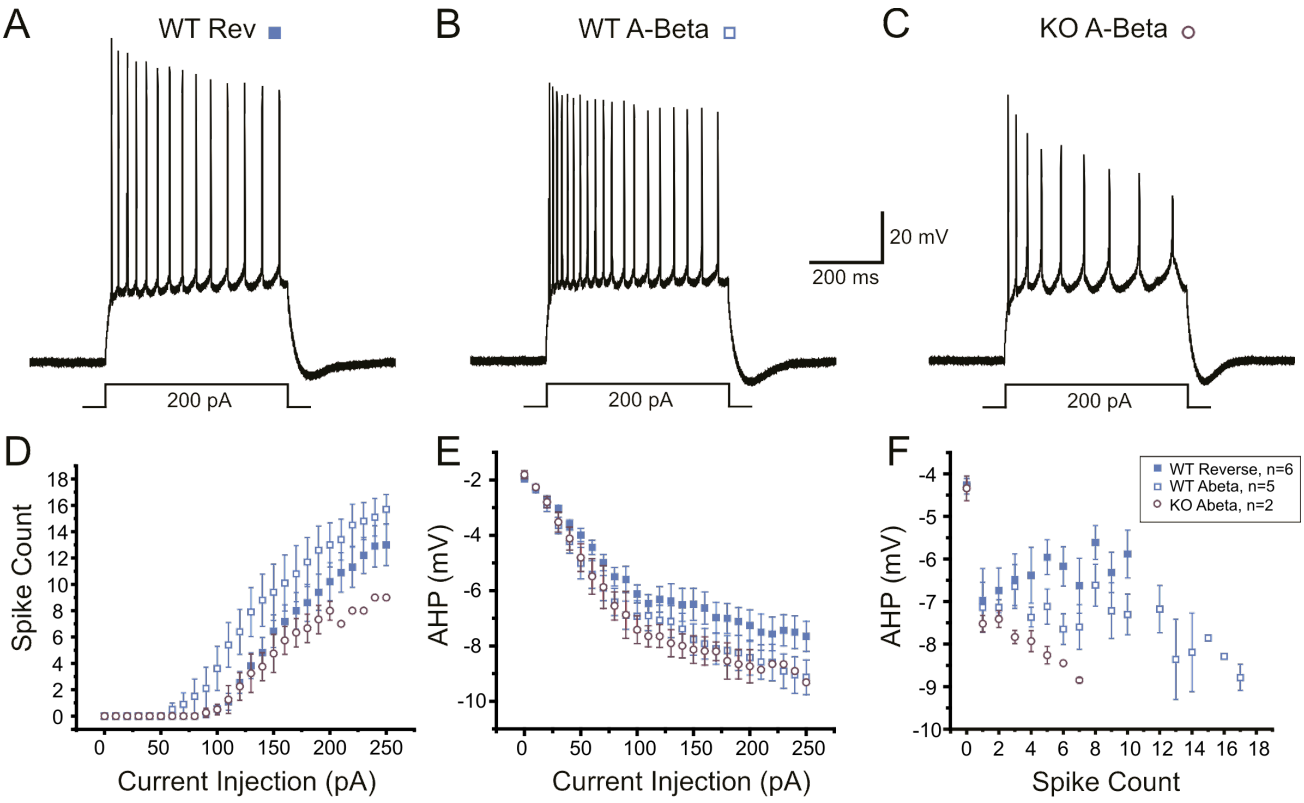

Figure 17. Action potential and after-hyperpolarization characteristics of $A\beta_{25-35}$ injected sig-1R KO mice Representative traces of action potential trains elicited by 200 pA step in WT vehicle treated (A) (n=6), WT $A\beta_{25-35}$ treated (B) (n=5) and KO $A\beta_{25-35}$ treated animals (C) (n=2). Analysis of spike count per current injection step (D), afterhyperpolarization amplitude per current injection step (E) and afterhyperpolarizaion amplitude compared to acition potential spike count (F). Error bars +/- SEM.

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Publisher	university of Ottawa
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Number of cartoons	2
Title or numeric reference of the portion(s)	The sigma-1 receptor chaperone as an inter-organelle signalling modulator. Figure 1, Figure 2.
Title of the article or chapter the portion is from	N/A
Editor of portion(s)	N/A
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The requesting person/organization	Kieran McCann
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