SELECTIVE ACTIVATION OF THE SK1 SUBTYPE OF SMALL CONDUCTANCE CA^{2+} ACTIVATED K^{+} CHANNELS BY GW542573X IN C57BL6J MICE IMPAIRS HIPPOCAMPAL-DEPENDENT MEMORY

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Robert W. Stackman, Jr., Department of Psychology, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Arts.

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ABSTRACT

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Title: Selective Activation of the SK1 Subtype of Small Conductance Ca^{2+} Activated K^{+} Channels by GW542573X in C57BL6J Mice Impairs Hippocampal-dependent Memory

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SK channels are small conductance Ca^{2+}-activated K^{+} channels expressed throughout the CNS. SK channels modulate the excitability of hippocampal CA1 neurons by affecting afterhyperpolarization and shaping excitatory postsynaptic responses. Such SK-mediated effects on activity-dependent neuronal excitability and synaptic strength are thought to underlie the modulatory influence of SK channels on memory encoding. Here, the effect of a new SK1 selective activator, GW542573X, on hippocampal-dependent object memory, contextual and cued conditioning, and trace fear conditioning was examined. The results showed that pre but not post-training systemic injection of GW542573X impaired object memory and trace fear memory in mice 24 h after training. Contextual and cued fear memory were not disrupted. These current data suggest that activation of SK1 subtype-containing SK channels impairs long-term memory. These

results are consistent with converging evidence that SK channel activation suppressed behaviorally triggered synaptic plasticity necessary for encoding hippocampal-dependent memory.

DEDICATION

This manuscript is dedicated to the memory of my brother, Leonard Joseph Rice, Jr. (Lenny), for his unwavering faith in my ability. I also dedicate this to my son, Caden Decker Kuchera, and my daughter, Kelsa Ross Kuchera, for putting up with late rides, take out dinners, and many weekend work days, and to my daughter, Mikala Ann for all of her encouragement these many years. I also would like to thank my sister, Lenore Jane Rice (Loni), for all of her help and support, and my mother, Helen Ross Rice, for instilling in me the value of education. Finally, I dedicate this manuscript to the memory of my father, Leonard Joseph Rice, Sr., who always said “the greatest adventure of all is exploring the brain.”

SELECTIVE ACTIVATION OF THE SK1 SUBTYPE OF SMALL CONDUCTANCE CA^{2+} ACTIVATED K^{+} CHANNELS BY GW542573X IN C57BL6J MICE IMPAIRS HIPPOCAMPAL-DEPENDENT MEMORY

I. INTRODUCTION

Of the four subunits comprising the small conductance Ca^{2+}-activated K^+ (SK) channel family, three subunits (SK1, SK2, and SK3) are expressed throughout the CNS, and with particularly high expression in brain regions important for long-term memory (Adelman, Maylie, & Sah, 2012; Stocker, 2004). It has been established that SK channels containing SK2 and SK3 subunits constrain the intrinsic excitability of neurons by enhancing afterhyperpolarization (Adelman et al., 2012; Hammond et al., 2006; Stackman et al., 2002; Stocker, 2004), shaping glutamatergic synaptic potentials (Faber, Delaney, & Sah, 2005; Ngo-Anh et al., 2005), and limiting the induction of NMDA receptor-dependent synaptic plasticity (Stackman et al., 2002).

Behaviorally, SK channels have been shown to modulate learning and memory encoding (Hammond et al., 2006; McKay et al., 2012; Stackman et al., 2002; Vick, Guidi, & Stackman, 2010). However, the specific role of the SK1 subunit has not yet been determined due to the prior lack of gene-specific antibodies and agonists.

SK1 subunits are robustly expressed in the CA1 pyramidal neurons of the hippocampus (Adelman et al., 2012; Stocker, 2004). SK1 subunits are sited only in the plasma membrane in rodents when co-expressed with SK2 or SK3 (Adelman et al., 2012; Stocker, 2004).

Co-expressed and co-assembled SK1, SK2, and SK3 subunits form functional apamin-sensitive channels (Adelman et al., 2012; Stocker, 2004). However, SK1 are not apamin-sensitive, suggesting the overriding hypothesis that SK1 is a subunit of

heteromeric SK channels that bind specific interacting proteins to influence subcellular localization and physiological roles including synaptic transmission, plasticity, and memory encoding.

Here the effects of a new SK1 selective activator, GW542573X (Hougaard et al., 2009) on hippocampal-dependent object recognition memory, contextual and cued fear conditioning, and trace fear conditioning were examined in male C57BL6J mice.

1.1 Structure

Calcium-activated potassium channels are divided into three classes: big (BK), intermediate (IK), and small (SK), based on their conductance (Adelman et al., 2012). Small conductance (approximately 10 ps) calcium-activated potassium channels can be subdivided further by subunit into SK1, SK2, SK3, and SK4 (Adelman et al., 2012). SK channels are composed of four transmembrane proteins, with each subunit protein containing six transmembrane domains. A loop between the fifth and sixth domain forms the potassium ion filter (Adelman et al., 2012; Stocker, 2004). Four of these subunits form a tetramer with the pore facing the center of the channel (Adelman et al., 2012; Stocker, 2004). These tetramers can be either heteromeric assemblies of SK subunits or homomeric channels (Adelman et al., 2012; Stocker, 2004).

1.2 Gating Mechanisms

SK channels are voltage independent and are gated directly by intracellular concentrations of Ca^{2+}, which enter the cell through voltage-gated calcium channels, NMDA receptors, or is released from intracellular stores (Adelman et al., 2012; Kohler et al., 1996; Maylie, Bond, Herson, Lee, & Adleman, 2004). Located on the C-terminus of each transmembrane subunit is a calmodulin binding domain (CaMBD) to which

calmodulin is constitutively bound. As the intracellular Ca^{2+} concentration increases and Ca^{2+} binds to each of the four CaMBDs, the SK channel changes its conformation causing a rotation of the CaMBDs, which opens the channel gate (Adelman et al., 2012; Stocker, 2004).

1.3 Assembly and Trafficking

Calmodulin and Ca^{2+} availability are not only essential for gating, but also are required for channel assembly and trafficking (Adelman et al., 2012; Lee, Ngo-Anh, Bruening-Wright, Maylie, & Adelman, 2003; Stocker, 2004). The Ca^{2+}-sensitivity of SK channels is negatively regulated by the protein kinase CK2-mediated phosphorylation of the constitutively bound calmodulin, and positively regulated by the protein phosphatase PP2A (Adelman et al., 2012; Hougaard et al., 2009; Stocker, 2004). Ca^{2+}-independent interactions with calmodulin are required for surface expression of SK channels (Lee et al., 2003).

SK channels contain multiple phosphorylation sites (Adelman et al., 2012). These phosphorylation sites modulate SK channel trafficking and long-term potentiation (LTP) dependent trafficking in CA1 pyramidal neurons (Adelman et al., 2012).

1.4 Function

When activated, SK channels allow potassium ions to cross the cell membrane. Activation of SK channels, expressed in the soma, regulates the hyperpolarization that follows an action potential (Stocker, 2004). The afterhyperpolarization (AHP) is composed of three dissociable component currents: a fast component, IfAHP; a medium component, ImAHP; and a slow component, IsAHP (Bond et al., 2004). The ImAHP is apamin-sensitive, and thus is considered to be SK channel dependent. Bond et al. (2004)

found the ImAHP to be abolished in mice with genetic deletion of the SK2 subunit. Under conditions of repetitive synaptic stimulation, the activation of SK channels and the ImAHP constrains neuronal excitability and affects synaptic transmission (Adelman et al., 2012; Hammond et al., 2006; Stackman et al., 2002; Stocker, 2004).

In the axon, spikes that initiate in the initial axon segment are propagated toward the terminal where neurotransmitter is released (Clark, Monsivais, Branco, London, & Häusser, 2005). The SK channel's enhancement of the afterhyperpolarization can influence the duration of these spikes, along with neurotransmitter release (Ngo-Anh et al., 2005).

SK channels localized in dendritic spines are positioned along with NMDARs within the Ca^{2+} signaling domain of the glutamatergic synapse (Ngo-Anh et al., 2005). Activation of these SK channels can limit the induction of NMDA receptor-dependent synaptic plasticity (Stackman et al., 2002) and limit the firing frequency of glutamate-mediated excitatory post-synaptic potentials (Faber et al., 2005; Ngo-Anh et al., 2005).

SK channels also have been implicated in a wide range of disorders, making them a prime target for therapeutic approaches. These disorders include age-related memory deficits (Blank, Nijholt, Kye, Radulovic, & Spiess, 2003), Parkinson's disease (Pedarzani & Stocker, 2008), epilepsy (Kleiman-Weiner, Beenhakker, Segal, & Huguenard, 2009), and sleep (Adelman et al., 2012). Recent studies implicate SK channel expression and function in addiction circuitry showing alteration by alcohol, nicotine, and illicit drugs (Padula et al., 2015). SK channels also have been shown to be a target for therapeutic treatment of anxiety and depression (Terstappen et al., 2003).

1.5 Distribution and Localization

SK1 and SK2 are robustly expressed in the CA1 and CA3 pyramidal neurons of the hippocampus, while only moderate levels of SK3 are found in these areas (Stocker & Pedarzani, 2000). SK2 channels are expressed in the postsynaptic density of dendritic spines in close proximity of synaptic NMDARs (Lin et al., 2010). Homomeric SK2 channels are located in the axons and dendrites, while heteromeric SK1/SK2 channels are found in the soma and dendrites, but not in axons (Bowden, Fletcher, Loane, & Marrion, 2001; Stocker, 2004). SK1 channels are selectively colocalized with D class L-type channels, long lasting voltage dependent Ca^{2+} channels (Bowden et al., 2001; Stocker, 2004).

The subcellular localization of SK1 channels is mainly somatic in dissociated CA1 neurons with some inclusion in the basal and apical dendrites, but is excluded from axons (Bowden et al., 2001). Heteromeric SK1/SK2 channels are found on the plasma membrane and in dendritic spines (Stocker, 2004). Homomeric channels composed of SK1 subunits are not expressed in the rodent brain (Stocker, 2004). SK1 subunits are sited only in the plasma membrane in rodents when co-expressed with SK2 or SK3 (Stocker, 2004). Thus, SK1 subunits may regulate the physiological properties of trafficking of heteromeric SK channels.

1.6 Pharmacological Tools

Several pharmacological tools have been developed to target SK channels, including peptide toxins, organic compounds, and enhancers. The majority of peptide toxins are non-subtype-specific SK channel blockers (Pedarzani & Stocker, 2008). Peptide toxins include apamin, derived from bee venom, and scyllatoxin, derived from

scorpion venom (Pedarzani & Stocker, 2008). Organic compounds such as d-tubocurarine and quaternary salts of bicuculline, dequalinium, and cyclophane derivatives such as UCL1684 and UCL1848 also are non-subtype-specific blockers (Pedarzani & Stocker, 2008). Exceptions are tamapin, a scorpion toxin, and leiurotoxin-Dab, which preferentially block SK2 channels (Pedarzani & Stocker, 2008).

Several compounds that enhance SK channel activity by enhancing the Ca^{2+} sensitivity include a series of structurally similar compounds: 1-ethyl-2-benzimidazolinone (1-EBIO) and 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309) (Pedarzani & Stocker, 2008). Also included in SK channel enhancers is the structurally distinct trisubstituted pyrimidine cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine] (CyPPA) (Pedarzani & Stocker, 2008). Recently a new SK1 agonist, GW542573X, has been synthesized that enhances SK1 activity by a deep pore gating mechanism rather than by altering the channel's Ca^{2+} sensitivity.

1.7 Behavioral Effects of SK Channel Blockers, Peptide Toxin

One of the most widely used pharmacological tools to study SK channel function is the peptide toxin apamin, a highly selective SK blocker (Garcia et al., 1991). There have been a number of studies of the behavioral effects of apamin. Homomeric SK2 channels are the most sensitive to apamin while SK1 channels are the least sensitive (Adelman et al., 2012). SK3 channels demonstrate intermediate sensitivity (Adelman et al., 2012). Blocking apamin-sensitive SK channels in hippocampal CA1 neurons facilitated encoding of hippocampal-dependent spatial and non-spatial hippocampal-dependent memory (Stackman et al., 2002). Stackman et al. (2002) found that systemic apamin-treated mice required fewer trials to learn the location of a hidden platform in a

Morris water maze task, and took less time to encode object memory in an object-recognition task compared to saline-treated control mice. However, apamin did not affect memory consolidation in either task (Stackman et al., 2002). In Criado-Marrero, Santini, and Porter (2014), rats that received infralimbic infusions of apamin demonstrated reduced fear in extinction protocols, suggesting that blocking SK channels facilitated extinction memory recall. Blocking SK channels with apamin enhanced the encoding of contextual fear memory during 1CS/US conditioning, but had no effect on 3 CS/US encoding sessions (Vick et al., 2010). In nonhippocampal-dependent water maze tasks, apamin did not influence memory encoding (Stackman et al., 2002). Thus it appears that apamin may have differing effects on different memory stages and on hippocampal-dependent versus hippocampal-independent tasks.

1.8 Behavioral Effects of SK Channel Activators, EBIO and CyPPA

In contrast to increased neuronal excitability as a result of blocking SK channels, activating SK channels decreases neuronal excitability (Pedarzani et al., 2001). Overexpression of SK2 was shown to reduce long-term potentiation (LTP) and severely impair spatial and contextual memory encoding (Hammond et al., 2006; Stackman, Bond, & Adelman, 2008). EBIO and CyPPA facilitate activation of SK channels by increasing Ca^{2+} sensitivity (Hougaard et al., 2009). While all SK channel subtypes exhibit equivalent sensitivity to EBIO (Pedarzani et al., 2001), CyPPA demonstrates greater selectivity for activating SK3 and SK2 channels (Hougaard et al., 2009). EBIO is evidenced to increase both medium and slow AHPs, strongly reducing electrical activity (Pedarzani et al., 2001).

Both EBIO and CyPPA impaired object memory encoding (Vick et al., 2010). CyPPA produced stronger impairment of memory encoding than did EBIO, suggesting that EBIO impairment is the result of activation of SK2 and SK3 rather than SK1 (Vick et al., 2010). Both SK activators failed to significantly affect encoding of contextual or cued fear memory (Vick et al., 2010).

1.9 Targeting the SK1 Subunit

It has been established that SK channels containing SK2 and SK3 subunits constrain the intrinsic excitability of neurons by enhancing afterhyperpolarization (Stackman et al., 2002; Stocker, Krause, & Pendarzani, 1999), by shaping glutamatergic postsynaptic potentials (Faber et al., 2005; Ngo-Anh et al., 2005), and by limiting the induction of NMDA receptor-dependent synaptic plasticity (Stackman et al., 2002). However, the specific role of the SK1 subunit has not yet been determined due to the prior lack of gene-specific antibodies or selective pharmacological tools.

Co-expressed and co-assembled SKl/SK2 and SK2/SK3 subunits form functional apamin-sensitive channels (Stocker, 2004). However, SK1 subunits are not apamin selective (Stocker, 2004), suggesting the overriding hypothesis that SKl is a subunit of heteromeric SK channels that bind specific interacting proteins to influence subcellular localization and physiological roles including synaptic transmission, plasticity, and memory encoding. A new SK1 activator, GW542573X, demonstrates selectivity for SK1 and is virtually inactive on SK2 and SK3 (Hougaard et al., 2009). In contrast to EBIO and CyPPA, which have been shown to depend on the CaMBD in the C terminus for activation of SK2 and SK3, GW542573X does not act via CaMBD, but rather by a “deep pore” gating structure in the inner pore of transmembrane segment 5 (Hougaard et al.,

2009). GW542573X represents a useful tool for distinguishing the contribution of SK1-containing SK channels on behavior, physiology, learning, and memory.

1.10 Hippocampal Memory

There are three main components that comprise the hippocampal memory system: the cerebral cortex, parahippocampal gyrus, and the hippocampus (Burwell, Witter, & Amaral, 1995; Suzuki, 1996). Each of these principal components contributes differently to declarative memory, along with essential interactions between these areas (Eichenbaum, 2000). Studies on amnesics have led to the hypothesis that the hippocampus and the parahippocampal gyrus contribute to the organization of memory in the cortex (Eichenbaum, 2000). The main component pathways show similarity in rodents and in primates (Eichenbaum, 2000). The hippocampus is essential for declarative memory encoding and recollecting events set in a spatiotemporal context (Cohen & Eichenbaum, 1993; Eichenbaum, 2000). Sequences of events and places may be represented by hippocampal neuronal networks that compose episodic memories (Eichenbaum, 2000). Information encoded by these neurons includes unique conjunctions of events and places, along with common overlapping experiences (Eichenbaum, 2000). Studies show the hippocampus generates spatial mapping and navigation guidance (Jarrard, 1993; Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe, 1999; O'Keefe & Nadel, 1978). Early work by O'Keefe and Nadel (1978), among others (Jarrard, 1993; Morris et al., 1982; O'Keefe, 1999), demonstrated that hippocampal damage affects spatial learning. O'Keefe and Nadel (1978) and O'Keefe (1999) have shown evidence that certain hippocampal pyramidal neurons, or place cells, fire at particular locations within an animal's environment.

Studies using animal models to characterize the mechanisms for information processing and neural circuitry in memory have shown parallels between amnesic humans and hippocampal damaged rodents (Squire, 1992). Humans and animals with hippocampal damage show intact sensory and cognitive processes as well as motor and motivational processes (Eichenbaum, 2000). The role of the hippocampus appears to be limited to transition of immediate memory to permanent memory and to a specific memory domain (Eichenbaum, 2000). Human amnesics and animals that have a damaged hippocampal region display memory loss after delay but not in immediate memory (Gaffan, 1974). This also has been observed after damage isolated to the hippocampus in certain cases of spatial (Clark, Zola, & Squire, 2000; Zola et al., 2000) and non-spatial memory (Clark et al., 2000; Cohen et al., 2013; Kesner & Novak, 1982).

1.11 Object Recognition

Memory tests are used to analyze the contribution of discrete brain regions to distinct types of memory and to distinct memory processes (Huang & Hsueh, 2014). Many memory tasks require conditions that impose stress by exposure to aversive stimuli or restriction of sleep, food, or water. These stressors potentially could affect the natural neural response (Huang & Hsueh, 2014). Unlike these aversively motivated, or appetitive motivated tasks, the object recognition task is a spontaneous memory test derived from curiosity and is easily manipulated (Huang & Hsueh, 2014). In novel object recognition (NOR), a sample session is presented in which mice are allowed to explore two identical objects until a criterion of exploration is reached. After an imposed delay, a test session is presented in which one of the objects is replaced with a novel object. Due to the natural

curiosity of mice, it is assumed that mice with intact memory for the objects explored during the sample session will prefer to explore the novel object during the test session.

Clark et al. (2000) argued that the hippocampus was necessary for non-spatial delay dependent memory tasks. In an object recognition protocol, rats with lesioned hippocampi exhibited impaired memory when long delays (10 min, 1 h, 24 h), but not short delays (10 s, 60 s), were imposed between the sample session and test session. It was posited that in this spontaneous novelty preference task, the ability of hippocampal lesioned rats to appreciate novelty and discriminate between objects was not impaired, as demonstrated by intact short delay memory. However, the impairment exhibited in long delay suggested hippocampal inactivation impaired object recognition memory.

Hammond, Tull, and Stackman (2004) demonstrated that while other components of the hippocampal system, such as the perirhinal cortex, may be sufficient for object recognition memory over short delay, the hippocampus is involved regardless of the period of delay. Inactivation of the dorsal hippocampus by intracranial lidocaine before the sample session impaired object recognition memory after a 24 h delay but not after a 5 min delay. Cohen et al. (2013) reported that object memory encoding, consolidation, and retrieval critically depend upon intact, functional dorsal hippocampal neurons. The results further demonstrated that novel object recognition could be used to test non-spatial hippocampal dependent object memory. To demonstrate that NOR did not merely test object-in-context memory, mice were given three different sample sessions where they were presented with the same objects but in three different contexts. Twenty-four h after the last sample session, mice received infusions of either the GABA-A agonist muscimol or vehicle and 40 min later were tested for novel object preference.

Hippocampal inactivated mice exhibited impaired novel object preference compared to controls. These results together demonstrate that the rodent hippocampus contributes significantly to object-in-context memory and to object memory independent of context. The present studies used the object recognition task to examine the influence of activating SK1-containing SK channels using the selective activator GW542573X on non-spatial memory processes.

1.12 Contextual and Cued Fear Conditioning

Freezing is a rodent's natural response to fear (LeDoux, 1993). In contextual and delay cued fear conditioning, the mouse is presented with repeated pairings of a tone conditional stimulus (CS) that co-terminates with a brief foot shock, unconditional stimulus (US), in a distinct context. The neutral stimulus becomes a CS after it is paired repeatedly with the aversive US (Phillips & LeDoux, 1992). The lateral amygdala has been shown to be critical for associating the tone (CS) with the foot shock (US), while the association between context memory (CS) and the foot shock (US) has been shown to be hippocampal and amygdala-dependent (Kim & Fanselow, 1992; Phillips & LeDoux, 1992). The ability to recognize items just learned, or immediate memory, is intact in patients with damage to the hippocampal region (Squire, Knowlton, & Musen, 1993). Quinn, Wied, Ma, Tinsley, and Fanselow (2008) demonstrated that the hippocampus is involved in contextual fear conditioning when the conditioning is weak but not when conditioning is strong; i.e., with more trials or higher shock intensity. This is consistent with the previously mentioned study by Vick et al. (2010) where blocking SK channels with systemic apamin enhanced the encoding of contextual fear memory during 1CS-US conditioning, but had no effect on 3 CS-US encoding sessions. The contextual and cued

fear conditioning protocol was used to examine the influence of GW542573X on hippocampal and amygdala-dependent fear memory in naïve mice.

1.13 Trace Fear Conditioning

In trace fear conditioning, there is a temporal gap between the termination of the tone (CS) and the onset of the aversive stimulus (US). An association between the CS and the US develops across this temporal gap. During trace fear conditioning, mice gradually acquire an appropriately timed anticipatory conditioned freezing response. The strength and accuracy of this temporally guided fear memory then can be assessed during a test session, often presented 24 h after conditioning. Trace fear conditioning is used to examine neural mechanisms in hippocampal dependent learning (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998). McEchron et al. (1998) found that the hippocampus was necessary in order to associate and remember the CS and US when they are separated by a long trace interval. Furthermore, the fact that contextual and cued fear are conditioned concomitantly makes trace fear well suited for examining hippocampal-dependent learning and memory (McEchron et al., 1998). Trace conditioning is ideal for studying the hippocampus' overlapping spatial and non-spatial roles (McEchron et al., 1998). Moreover, this memory is dependent on hippocampal function in rodents and humans (Clark & Squire, 1998; Huerta, Sun, Wilsona, & Tonegawaa, 2000; McEchron et al., 1998). The trace fear conditioning protocol was used to assess the influence of SK1-containing SK channels on a well-characterized form of hippocampal-dependent temporal memory in naïve mice.

1.14 Hypotheses and Predictions

Here, the effect of GW542573X on hippocampal dependent object memory, contextual and cued fear conditioning, and trace fear conditioning in male C57BL6J mice was examined. The aim of this study was to explore for the first time how selective activation of SK1 influences behavior during novel object discrimination and during delay and trace fear conditioning in mice. Based on previous reports of memory effects of systemic activators and blockers of SK2 and SK3 channels, it was hypothesized that selective activation of SK1-containing channels would affect the encoding, but not consolidation, of hippocampal-dependent memory. It also was hypothesized that selective activation of SK1-containing channels would not affect hippocampal-independent memory. In order to test these hypotheses, three experimental methods were used. First, encoding and consolidation in a non-spatial memory task, using an object recognition protocol, were tested. Secondly, encoding in a contextual and cued (3 CS/US pairings) fear conditioning protocol was tested. Finally, encoding and consolidation in a trace fear conditioning (8 CS/US pairings) protocol were tested. The main prediction was that activation of SK1 would impair object memory and trace fear tone memory encoding, but not the encoding of contextual or cued fear memory or the encoding of trace fear contextual memory. It also was hypothesized that consolidation would not be affected by the activation of SK1 channels. The data demonstrate that SK1 activation impairs non-spatial object memory encoding and trace fear memory encoding. However, encoding in contextual and cued fear conditioning was not impaired. Memory consolidation was not affected.

2. MATERIALS AND METHODS

2.1 Subjects

Subjects were 20 (7-8 week old) male C57BL/6J mice (Jackson Labs, Bar Harbor, ME). Mice were housed in groups of four per standard polycarbonate cage with ad libitum access to food and water. Mouse cages were maintained in a temperature- and humidity-controlled colony room with a 12-h/12-h light/dark cycle with lights on at 7:00 AM. Bedding changes occurred 1/week on a day that was convenient to the experimental protocol (i.e., bedding was changed before behavioral training was initiated or delayed until completion of a given behavioral protocol). All behavioral testing took place during the light phase from 12:00 PM to 5:00 PM. All procedures were conducted in accordance with the guidelines as described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Florida Atlantic University.

2.2. Drugs

GW542573X was purchased from Tocris (Ellisville, MO). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO). A 4% solution of DMSO in sterile saline served as the vehicle in all GW542573X (15.00 mg/kg,) experiments. GW542573X was freshly prepared at the time of administration.

2.3 Preliminary Procedures

Procedures began at least 1 week after arrival of mice into the vivarium. On the first day of behavioral testing (day 1) each mouse was habituated to the holding room for

at least 1 h; on day 2 and 3, each mouse was placed individually in a clean, empty polycarbonate holding cage for a 10-min period then returned to its home cage.

2.4 Preliminary Testing for Object Recognition Open Field

2.4.1 Habituation

On day 4 and 5, each mouse was restrained momentarily and received an abdominal subcutaneous prick with a 26 gauge needle before being placed in the polycarbonate cage for 25 min. This procedure acclimated mice to the handling necessary for giving systemic drug injections. Mice then were returned to their home cages.

2.4.2 Open Field Test

Dose-dependent analysis of the effects of systemic GW542573X on locomotor activity previously was conducted to determine appropriate dosage range, administration and testing interval, and drug side effects. These results revealed that administration of GW542573X to male C57BL/6J mice induced a brief period of significant suppression of locomotor activity, which recovered to baseline levels by 25 min after injection. Thus, for all of the present experiments mice, received 15mg/kg of GW542573X 25 min prior to the protocol being implemented.

2.4.3 Statistical Analysis

Activity was recorded and automatically scored using Noldus Ethovision XT (version 7.0). An unpaired Student's *t*-test was conducted to analyze treatment differences in open field distance traveled and velocity. Distance traveled was defined as the sum of recorded movement of the center point of the mouse in cm over the duration of the trial.

2.5 Experiment 1: Object Recognition Memory

2.5.1 Habituation

On day 4 and 5, each mouse was restrained momentarily and received an abdominal subcutaneous prick with a 26 gauge needle before being placed in the polycarbonate cage for 25 min. Mice then were given a 10 min habituation session in a conventional object memory symmetrical square arena (37.5 x 37.5 x 50 cm, constructed of white ABS) before being returned to their home cage.

2.5.2 Sample Session with Pre-sample Administration of GW542573X

On day 6, mice received GW542573X (15 mg/kg, IP) or DMSO (4%, IP) vehicle before being placed in the polycarbonate cage for 25 min. Mice then were placed in the familiar arena for 10 min and were permitted to explore two identical novel objects that were placed in opposite corners within the arena. The objects, two identical threaded table feet, each mounted on a clear acrylic base (6x6 cm), were prescreened for preference and discrimination (Cohen et al., 2013). The trial ended once the mouse had accumulated 30 s exploration time per object, or 38 s exploration time for one object. Mice that failed to achieve the exploration criteria within 10 min were removed from the study. The latency to reach the exploration criteria was recorded and automatically scored for each mouse using Noldus Ethovision XT (version 7.0). Object exploration was defined as time spent with the head oriented toward and within 2-3 cm of the object. Mice then were placed back into their home cage.

2.5.3 Object Recognition Test

Twenty-four h following the sample session, mice received a subcutaneous prick to simulate IP injection. Mice were placed in a polycarbonate cage for 25 min, then given

a 5 min test session in the same arena as the sample session. One of the previously encountered objects was replaced with a novel one, a purple toy gorilla, approximately the same size as the familiar object. The novel object also was prescreened for preference and discrimination (Hammond et al., 2004). In order to avoid place preference, the familiar object that the mouse spent the least amount of time exploring was the object that was replaced. The arena was wiped down with 10% ethanol between each session to eliminate any odor cues that may be present. Objects were cleaned with 10% ethanol to decrease any olfactory cues from the familiar object during test session.

2.5.4 Habituation

On day 4 and 5, each mouse placed in a polycarbonate cage. Mice then were given a 10 min habituation session in a conventional object memory symmetrical square arena (37.5 x 37.5 x 50 cm, constructed of white ABS). Before being returned to their home cage, each mouse was restrained momentarily and received an abdominal subcutaneous prick with a 26 gauge needle.

2.5.5 Sample Session with Post-sample Administration of GW542573X

On day 6, mice were placed in a polycarbonate cage, then were placed in the familiar arena for 10 min and were permitted to explore two identical novel objects that were placed in opposite corners within the arena. The objects, two identical threaded table feet, each mounted on a clear acrylic base (6x6 cm), were prescreened for preference and discrimination (Cohen et al., 2013). The trial ended once the mouse had accumulated 30 s exploration time per object, or 38 s exploration time for one object. Mice received GW542573X (15 mg/kg, IP) or DMSO (4%, IP) vehicle immediately upon removal from the arena. Mice then were placed back into their home cage. Mice that

failed to achieve the exploration criteria within 10 min were removed from the study. The latency to reach the exploration criteria was recorded and automatically scored for each mouse using Noldus Ethovision XT (version 7.0). Object exploration was defined as time spent with the head oriented toward and within 2-3 cm of the object.

2.5.6 Object Recognition Test

Twenty-four h following the sample session, mice were placed in a polycarbonate cage and then given a 5 min test session in the same arena as the sample session. One of the previously encountered objects was replaced with a novel one, a purple toy gorilla, approximately the same size as the familiar object. The novel object also was prescreened for preference and discrimination (Hammond et al., 2004). In order to avoid place preference, the familiar object that the mouse spent the least amount of time exploring was the object that was replaced. The arena was wiped down with 10% ethanol between each session to eliminate any odor cues that may be present. Objects were cleaned with 10% ethanol to decrease any olfactory cues from the familiar object during test session.

2.5.7 Statistical Analysis

Object exploration was determined for each mouse based on the manual scoring of behavior from the sample and test sessions. Object memory was inferred from discrimination ratio scores, calculated for each mouse as the difference in time spent exploring the novel object minus the time spent exploring the familiar object divided by the total time spent exploring objects (TNovel object – TFamiliar object / TNovel object + TFamiliar object). An unpaired Student's *t*-test was conducted to analyze treatment differences in discrimination ratio, total object exploration, and latency to criteria. In addition, distance traveled was tracked by visual tracking software Noldus Ethovision XT

(version 7.0) and analyzed to determine drug induced response. An unpaired Student's *t*-test was conducted to analyze treatment differences in distance traveled and velocity. Distance traveled was defined as the sum of recorded movement of the center point of the mouse in cm over the duration of the trial.

2.6 Experiment 2: Delay Fear Conditioning

2.6.1 Materials

Delay fear conditioning was performed using the MED Associates Near-Infrared Video Fear Conditioning System (Georgia, VT) composed of four identical rectangular chambers (30.5 cm by 24.1 cm by 21 cm) constructed of brushed aluminum side walls and clear Plexiglas front, back, and top walls. An overhead white house light and an infrared light illuminate each chamber, and a speaker attached to the right side wall of each chamber delivers the tone stimuli. The chamber floor is constructed of parallel stainless steel rods (36 rods, 3.2 mm dia, 7.9 mm apart) designed for mice and connected to a scrambled shock generator. Each chamber is housed inside a larger sound-attenuating cabinet, which has a ventilation fan in the right side wall used to provide background noise. A near-infrared FireWire video camera, mounted on the left front door of each noise-attenuating cabinet, was used to acquire mouse behavior. Before each trial, the chamber floors was cleaned thoroughly with a 10% ethanol solution, then with 1% LiquiNox (White Plains, New York) to remove olfactory cues.

Freezing was recorded and automatically scored during pre-exposure, conditioning, tone, and context sessions to assess the strength of fear memory. The automatic software program operationally defines freezing as the duration of time, greater than 0.6 s, during which fewer than 20 pixels of each video frame were detected to have

changed. The video capture rate was set at 30 fps, which results in freezing being recorded computationally after less than 20 pixels of motion per frame over the time course of 18 frames.

2.6.2 Context Pre-Exposure

On day 4, each mouse received an abdominal subcutaneous prick with a 26 gauge needle before being placed in the polycarbonate cage for 25 min. Mice then were habituated for 5 min to a fear chamber wiped with 1% vanilla extract to provide an odor cue.

2.6.3 Contextual and Cued Fear Conditioning

On day 5, mice received GW542573X (15 mg/kg, IP) or DMSO (4%, IP) vehicle before being placed in the polycarbonate cage for 25 min. After 25 min, mice were given a sample session where they were returned to the same chamber with same odor cue and exposed to a 3 conditioned stimuli/unconditioned stimuli pairings. After 60 s, a 5000 Hz, 90 dB tone (CS) was presented for 30 s, co-terminating with a 1 s 0.5 mA foot shock (US). The CS-US pairing was presented twice more with a 120 s interval between tone onset. Mice were removed from the chamber 60 s after the presentation of the third CS-US pairing.

2.6.4 Tone Test

To assess cued fear memory retention on day 6, mice received skin pricks to simulate IP injections. After 25 min mice were given a 2 tone test in modified chambers. After a 60 s delay, a 5000 Hz, 90 dB tone (CS) for 30 s played followed by a 120 s interval. A second 5000 Hz, 90 dB tone (CS) then played for 30 s. Mice were removed from the chamber 30 s after termination of the second tone. Context of the chamber was

changed by installing a teepee roof, a floor shield, 1% orange extract as odor cue, and filtered light in order to assess the strength of fear memory based on the CS.

2.6.5 Context Test

Approximately 1 h later, contextual fear memory was tested during a 5 min session in the original chamber with no tone or shock presented and with identical contextual cues. The walls and floor of the chamber were wiped down with 10% ethanol between each session to eliminate any odor cues that may be present. During the delay between sessions, mice were placed back into their polycarbonate cages.

2.6.6 Statistical Analyses

Total percent freezing responses were calculated for each animal for the 5-min pre-exposure and context test sessions, and analyzed using an independent groups *t*-test. Percent freezing scores were analyzed for each animal during the conditioning session using a repeated-measures ANOVA with the between-subjects factor being treatment (GW542573X or DMSO) and the within-subjects factor being time bin (the first 60 s pre-CS period), and each 60 s CS-US period (comprising the 30 s tone, plus the 30 s that followed), with post-hoc multiple comparisons tests where appropriate. Tone test freezing response was analyzed by repeated-measures ANOVA with the between-subjects factor being treatment (GW542573X or DMSO) and the within-subjects factor of % freezing during the 60 s pre-CS interval, the 30 s CS period, and the 30 s post-CS interval.

2.7 Experiment 3: Trace Fear Conditioning

2.7.1 Materials

Trace fear conditioning was performed using the same apparatus described in

Experiment 2. Freezing was recorded and automatically scored to assess the strength of fear memory using the same software program described previously.

2.7.2 Trace Fear Conditioning With Pre-Conditioning Administration of GW542573X

On day 4, mice received GW542573X (15 mg/kg, IP) or DMSO (4%, IP) vehicle before being placed in the polycarbonate cage for 25 min. After 25 min, mice underwent a trace fear conditioning session, where they were placed individually into the chamber with the same odor cue and received 8 CS-US pairings. Thirty s after placement in the chamber, a 5000 Hz, 90 dB, 15 s tone (CS) was played followed by a 15 s delay co-terminating with a 1 s, 0.5 mA foot shock (US). A 120 s interval was imposed between tone presentations. Mice were removed from the chamber 60 s after the presentation of the 8^{th} US.

2.7.3 Tone Test

To assess memory retention after a 24 h delay, mice received skin pricks to simulate IP injections before being placed in the polycarbonate cage for 25 min. After 25 min mice were given a 2 tone test in modified chambers where a 5000 Hz, 90 dB tone (CS) was presented for 15 s after an initial 60 s delay. A second 5000 Hz, 90 dB tone (CS) played for 15 s following a 120 s interval. Mice were removed 30 s after termination of the tone. Context of chamber was changed by installing a teepee roof, a floor shield, 1% acetic acid, and filtered lighting.

2.7.4 Context Test

Approximately 1 h later, contextual fear memory was tested during a 5 min session in the chamber with no tone or shock presented and with identical contextual cues

to the original sample session, including 1% liquinox as scent. The walls and floor of the chamber were wiped down with 10% ethanol between each recording session to eliminate any odor cues that may be present. During the delay between sessions, mice were returned to their home cages.

2.7.5 Trace Fear Conditioning With Post-Conditioning Administration of GW542573X

On day 4, mice were placed in a polycarbonate cage, then underwent a trace fear conditioning session where they were placed individually into the chamber with the same odor cue, and received 8 CS-US pairings. Thirty s after placement in the chamber, a 5000 Hz, 90 dB, 15 s tone (CS) was played followed by a 15 s delay co-terminating with a 1 s, 0.5 mA foot shock (US). A 120 s interval was imposed between tone presentations. Mice were removed from the chamber 60 s after the presentation of the 8^{th} US. Immediately upon removal from the chamber, mice received GW542573X (15 mg/kg, IP) or DMSO (4%, IP) vehicle before being returned to their home cage.

2.7.6 Tone Test

To assess memory retention after a 24 h delay, mice were given a 2 tone test in modified chambers where a 5000 Hz, 90 dB tone (CS) was presented for 15 s after an initial 60 s delay. A second 5000 Hz, 90 dB tone (CS) played for 15 s following a 120 s interval. Mice were removed 30 s after termination of the tone. Context of chamber was changed by installing a teepee roof, a floor shield, 1% acetic acid, and filtered lighting.

2.7.7 Context Test

Approximately 1 h later, contextual fear memory was tested during a 5 min session in the chamber with no tone or shock presented and with identical contextual cues

to the original sample session, including 1% liquinox as scent. The walls and floor of the chamber were wiped down with 10% ethanol between each recording session to eliminate any odor cues that may be present. During the delay between sessions, mice were returned to their home cages.

2.7.8 Statistical Analyses

Total percent freezing responses were calculated for each animal for the 5-min pre-exposure and context test sessions, and analyzed using an independent groups *t*-test. Percent freezing scores were analyzed for each animal during the conditioning session using a repeated-measures ANOVA with the between-subjects factor being treatment (GW542573X or DMSO) and the within-subjects factor being time bin (the first 60 s pre-CS period), and each 15 s CS period or CS post 1 (the 15 s following the CS) with post multiple comparisons tests where appropriate. Tone test freezing responses were analyzed by repeated-measures ANOVA with the between-subjects factor being treatment (GW542573X or DMSO) and the within-subjects factor of % freezing during the 60 s pre-CS interval, the 15 s CS period, the first 15 s post-CS interval, and the second 15 s post-CS interval. To examine whether GW542573X differentially influenced the perception of the aversive foot shock stimulus, an activity burst was analyzed for each mouse by comparing the motion score for the first shock interval, 1 s, + 1 s post shock to a 2 s pre-shock interval (mean shock/mean no shock). An unpaired Student's *t*-test then was conducted to analyze treatment differences in the mean activity burst.

3. RESULT

3.1 Pre-Sample Session Administration of GW542572X Impairs Encoding of Object Recognition Memory

In order to examine the influence of SK1 activation on encoding of non-spatial memory, an object memory recognition task was employed. Activating SK1 by systemic injection of the SK1 agonist GW542573X before the sample session led to impaired object memory in mice 24 h later.

3.1.1 Object Recognition Sample Session

Following two, 10 min habituation sessions, 24 h and 48 h earlier respectively, mice received GW542573X or vehicle 25 min prior to the sample session. During the sample session mice were given 10 min in order to acquire object exploration of 30 s per object or 38 s total on both objects. Of the 34 mice that underwent novel object recognition testing, 8 mice were removed from further analysis. Seven mice did not reach the exploration criteria during the 10 min object sessions. One mouse was removed for treatment error. An unpaired Student's *t*-test was conducted to analyze treatment differences in latency to acquire, total object exploration, distance traveled, and velocity.

Mice treated with GW542573X, $n = 11$, acquired the sample object exploration criterion in a similar latency as vehicle-treated mice, $n = 13$, $t(24) = -.04$, *ns* (see Figure 1A). Total exploration time between groups was not significantly different between treatment conditions, $t(24) = 158$, *ns* (see Inset Figure 1.A).

Distance traveled during the sample session was not significantly different between the vehicle-treated group and the GW542573X-treated group, $t(24) = .387$, *ns*. Velocity between groups also was non-significant between groups, $t(24) = 0.23$, *ns* (see Figure 1.B).

3.1.2 Object Recognition Memory Test Session

Twenty-four h later, mice received a 5 min test session with one familiar object and one novel object. In order to avoid place preference the object with less exploration time from the previous day was replaced with the novel object. An unpaired Student's *t*-test was conducted to analyze novel object preference as determined by discrimination ratio (TNov-TFam)/(TNov + TFam). Mice that received pre-sample GW542573X exhibited significantly less preference for exploring the novel object during the test session in contrast to mice that had received pre-sample vehicle, $t(24) = 3.88$, $p = 0.009$ (see Figure 2). Follow up analyses revealed that both treatment groups exhibited discrimination ratio scores that were significantly above chance: vehicle, $t(11) = 9.96$, $p > 0.05$, and GW542573X-treated, $t(11) = 3.25$, $p > 0.05$.

The results suggest that the SK1 activator disrupted the encoding of object memory without affecting the motivation to explore objects as indicated by the lack of difference in latency to acquire and total object exploration during the sample session.

3.2 Post-Sample Session Administration of GW542572X Does Not Affect Consolidation of Object Recognition Memory

In order to examine the influence of SK1 activation on consolidation of non-spatial memory the same object memory recognition task was employed. Activating SK1

by systemic injection of the SK1 agonist GW542573X post-sample did not impair object memory in mice 24 h later.

3.2.1 Object Recognition Sample Session

Mice were randomly assigned to the respective post-sample treatment groups and were individually placed into the familiar arena for a sample session using the same procedure. Of the 18 mice that underwent novel object recognition testing, one mouse was removed from further analysis due to failure to reach the sample session object exploration criteria within the required 10 min. Mice marked for treatment with GW542573X acquired the sample object exploration criterion in a similar latency as mice marked for treatment with vehicle, $t(15) = 0.12$, *ns*.

Distance traveled during the sample session was not significantly different between the future treatment groups: vehicle, $n = 9$, and GW542573X, $n = 8$, $t(15) = 0.79$, *ns*. Velocity between groups also was non-significant, $t(15) = 0.04$, *ns*.

3.2.2 Object Recognition Memory Test Session

Mice that received post-sample vehicle, $n = 9$, exhibited a significant novel object preference, as inferred from the discrimination ratio, $t(8) = 5.88$, $p < 0.00$. Similarly, mice treated with post-sample GW542573X demonstrated a significant discrimination between test session objects, $t(7) = 4.75$, $p < 0.00$. Additionally, the vehicle-treated and GW542573X-treated mice displayed similar test session discrimination, $t(15) = 0.38$, *ns* (see Figure 3). These results suggest that activating SK1 immediately post-sample did not disrupt the consolidation of object memory.

3.3 Pre-Conditioning Administration of GW542572X Does Not Affect Encoding of Contextual or Cued Fear Memory

The influence of SK1 activation on encoding contextual and cued fear memories was examined. Systemic administration of the SK1 agonist GW542573X or vehicle either pre-conditioning or post-conditioning did not impair contextual or cued fear memory expression 24 h later.

3.3.1 Context Pre-Exposure

Mice were randomly assigned to one of the two treatment conditions (n = 6/condition), and then were placed into the conditioning chamber for a 5-min context pre-exposure session. Inset Figure 4.C shows no significant difference in pre-exposure % freezing between the two future treatment groups $t(10) = 1.33$, *ns*.

3.3.2 Contextual and Cued Fear Conditioning

Mice received GW542573X (15 mg/kg, $n = 6$) or vehicle ($n = 6$) before being placed in a polycarbonate holding cage for 25 min. After 25 min, mice were returned to the same conditioning chamber with the same odor cue and were exposed to a 3 CS-US pairing. Analysis of % freezing measures from the conditioning session with a repeated measures ANOVA yielded no significant effect of treatment, $F(1,10) = 0.47$, *ns*, a significant time bin effect $F(3,30) = 31.65$, $p < 0.001$, but no significant treatment * time bin interaction, $F(3,30) = 0.49$, *ns* (Figure 4.A).

3.3.3 Cued Fear Memory Test

Twenty four h after conditioning, each mouse was placed in a modified chamber and the strength of the cued fear memory was assessed. Mice that had received pre-conditioning GW542573X exhibited tone-elicited freezing that was comparable to that of

the vehicle-treated group. A two-factor repeated measures ANOVA yielded no significant effect of treatment, $F(1,10) = 0.72$, *ns*; a significant effect of time bin, $F(2,20) = 153.41$, $p < 0.001$; but no significant treatment * time bin interaction, $F(2,20) = 0.69$, *ns* (see Figure 4.B).

3.3.4 Contextual Memory Test

Mice were returned to the original conditioning chamber to assess the strength of the contextual fear memory. Mice that had received pre-conditioning GW542573X exhibited freezing responses during the context test that were comparable to those of the vehicle-treated group. Total % freezing during the context test 24 h after conditioning was not significantly different between pre-conditioning treatment groups, $t(10) = 0.74$, *ns* (see Figure 4.C). This was not surprising, since GW542573X had not yet been administered when context memory was encoded. The results suggest that activating SK1 did not disrupt encoding of contextual or cued fear memory. Given the results of the encoding protocol, consolidation was not examined.

3.4 Pre-Conditioning Administration of GW542572X Impairs Encoding of Trace Fear Memory

The influence of SK1 activation on encoding of hippocampal dependent temporal memory was determined with a trace fear conditioning protocol. Activating SK1 by systemic injection of the SK1 agonist GW542573X pre-conditioning led to impaired tone, but not contextual, memory in mice 24 h later.

3.4.1 Trace Fear Conditioning

Mice received systemic injection of the SK1 agonist GW542573X or vehicle, 25 min prior to the start of the trace conditioning session. Of the 26 mice that underwent

trace fear conditioning, two were removed from further analysis; one was removed because of a physical defect and the other was removed after a treatment error. As shown in Figure 5.A, mice that received pre-conditioning GW 542573X (15 mg/kg; $n = 11$) exhibited less freezing during each CS/US pairing compared to vehicle-treated mice ($n = 13$). The two-factor repeated measures ANOVA on % freezing in all time bins yielded no significant overall treatment effect, $F(1,22) = 2.23$, *ns*; a significant time bin effect, $F(48, 1056) = 21.39$, $p < 0.001$; but no significant treatment * time bin effect, $F(48, 1056) = 0.78$, *ns*. Holm-Sidak was used for pairwise comparisons, revealing significant differences between time bins.

Additional analyses were conducted to evaluate the treatment effect on the acquisition of anticipatory freezing responses during the trace conditioning session. As shown in Figure 5.B, a two-factor repeated measures ANOVA on % freezing in each CS time bin yielded no significant overall treatment effect, $F(1, 22) = 2.38$, *ns*, but a significant time bin effect, $F(24, 528) = 19.5$, $p < 0.001$. Holm-Sidak was used for pairwise comparisons, revealing significant differences between CS1 and subsequent CS time bins. There was no significant treatment * time bin effect, $F(24, 528) = 0.66$, *ns*. Additionally a two-factor repeated measures ANOVA yielded no significant overall treatment effect between 15 s post-CS time bins, $F(1,22) = 1.81$, *ns*, but a significant time bin effect $F(7, 154) = 14.73$, $p < 0.001$. Holm-Sidak was used for pairwise comparisons revealing significant differences between CS1 post 1 and subsequent CS post 1 time bins. There was no significant treatment * time bin effect, $F(7, 154) = 0.66$, *ns*. While there was no significant overall treatment effect between groups, $F(1,22) = 2.38$, *ns,* there was

a trend showing that GW542573X-treated mice exhibited less freezing during each CS and during each CS post-1 compared to vehicle-treated mice.

3.4.2 Trace Fear Memory Test

The strength of the trace fear memory was assessed 24 h after conditioning by placing the mice in a modified chamber and after 60 s, a 15-s CS was presented. During the trace tone test session (see Figure 5.C), mice that had received pre-conditioning GW542573X exhibited significantly less freezing during post CS 1compared to vehicle-treated mice. A two-factor repeated measures ANOVA on % freezing during pre-CS, CS, 15 s post-CS, and 30 s post-CS yielded a significant treatment effect, $F(1,22) = 5.17$, $p = 0.03$; a significant effect of time, $F(1,22) = 65.59$, $p = 0.000$; and a significant time bin * treatment interaction, $F(1,22) = 5.53$, $p = 0.03$. Mauchly's test of sphericity indicated that sphericity was met, *ns*. Levene's test of equality revealed variances between groups was not significant, $p > 0.05$. Post hoc analysis with Holm-Sidak tests to determine pairwise comparisons revealed a significant difference between pre-CS and CS and between CS and post-CS1. Pairwise comparisons also revealed a significant treatment * time bin effect between treatment groups during 15 s post-CS time bin. A Student's *t*-test revealed significant difference between the GW542573X-treated group and the vehicle-treated group during post CS1, $t(22) = 2.34$, $p = 0.03$.

3.4.3 Trace Fear Context Test

There were no significant differences in total % freezing during the context test between pre-conditioning treatment groups $t(9) = -0.86$, *ns* (See Figure 5.D**)**

3.4.4 Activity Burst

In order to confirm that GW542573X did not affect the perception of the aversive foot shock stimulus, the velocity of the visible unconditional response (UR) to the first shock during trace conditioning was assessed. Total % freezing during pre-CS and post-CS was not significantly different between the mice that received pre-conditioning vehicle or GW542573X, pre-CS, $t(12) = 0.49$, *ns*, or post-CS, $t(12) = 0.95$, *ns* (see Inset Figure 5.C).

3.5 Activation of SK1 with GW542572X Does Not Impair Consolidation of Trace Fear Memory

A second cohort of mice received trace fear conditioning using the identical procedure as above, and then received systemic GW542573X or vehicle immediately after trace conditioning was completed. This experiment was conducted to test the influence of SK1 activation on consolidation of non-spatial memory on hippocampal dependent trace fear memory. SK1 activation was examined using contextual and tone trace fear protocols.

3.5.1 Influence of Post-Conditioning Administration of GW542573X on Trace Fear Conditioning

Mice were randomly assigned to either of the respective post-conditioning treatment conditions and then underwent trace fear conditioning as described previously. There were no differences in acquisition of the appropriate conditioned freezing response during trace fear conditioning between the future treatment groups. A two-factor repeated measures ANOVA on pre-CS, all CS, and CS post 1 time bins yielded no significant treatment effect, $F(1,16) = 0.28$, *ns* (Figure 6.A); a significant difference between time

bins, $F(16, 256) = 19.21$, $p < 0.001$; but no significant time bin * treatment interaction, $F(16, 256) = 0.69$, *ns*.

Additional analyses were conducted to evaluate the treatment effect on the acquisition of anticipatory freezing responses during the trace conditioning session. A two-factor repeated measures ANOVA yielded no significant overall treatment effect between CS time bins, $F(1, 16) = 1.14$, *ns*, a significant time bin effect $F(8,128) = 19.17$, $p < 0.001$. Holm-Sidak was used for pairwise comparisons, revealing significant differences between CS1 and subsequent CS time bins. There was no significant treatment * time bin effect, $F(8,128) = 0.76$, *ns*. Additionally, a two-factor repeated measures ANOVA yielded no significant overall treatment effect between 15 s post-CS time bins, $F(1, 16) = 0.06$, *ns*, a significant time bin effect $F(7, 110) = 14.65$, $p < 0.001$. Holm-Sidak was used for pairwise comparisons revealing significant differences between CS1 post 1 and subsequent CS post 1 time bins. There was no significant treatment * time bin effect, $F(7,110) = 0.27$, *ns* (Figure 6.B).

3.5.2 Trace Fear Memory Test

During trace tone testing, activating SK1 by systemic injection of the SK1 agonist GW542573X post-conditioning did not lead to impaired tone trace memory in mice. A two-factor repeated measures ANOVA yielded no significant overall treatment effect, $F(1, 22) = 1.24$, *ns*, but a significant time bin effect, $F(3,48) = 27.88$, $p < 0.001$. Holm-Sidak was used for pairwise comparisons revealing significant differences between pre-CS and all subsequent time bins. There was no significant treatment * time bin effect, $F(3,48) = 0.54$, *ns* (Figure 6.C).

3.5.3 Trace Fear Context Test

There were no significant differences in total % freezing during the context test between post-conditioning treatment groups $t(16) = 0.88$, *ns* (see Figure 6.D). Activating SK1 by systemic injection of the SK1 agonist GW542573X post-conditioning did not lead to impaired tone, or contextual, memory in mice 24 h later. These results suggest that activating SK1 did not disrupt consolidation of tone trace fear memory or contextual trace fear memory.

4. DISCUSSION

The contribution of SK1 to learning and memory previously has not been examined due to a lack of SK1 selective activators and blockers. Here, the effects of a new SK1 selective agonist, GW542573X, were tested on hippocampal-dependent object memory, contextual and cued fear conditioning, and trace fear conditioning in male C57BL6J mice. The results demonstrated that activating SK1 by systemic injection of GW542573X pre-sample, but not post-sample, led to impaired object memory in an object recognition protocol. Pre-conditioning treatment did not disrupt contextual or cued fear memory. Lastly, activating SK1 pre-conditioning in a hippocampal-dependent trace fear memory protocol resulted in impaired temporal memory. Post-conditioning activation of SK1 did not result in impairment of temporal memory. These results suggest that SK1 activation impairs memory encoding but not consolidation in hippocampal-dependent object memory and trace fear memory, but not memory encoding in contextual and cued fear.

The results obtained from activating SK1 and examining its behavioral effect on hippocampal-dependent memory encoding is consistent with current research on SK activation and blocking apamin sensitive SK channels. However, the interesting inconsistences between SK channel activation versus overexpression, activation versus blocking, and weak versus strong memory encoding suggests an independent physiological role for SK1 in synaptic transmission, plasticity, and memory encoding, and are discussed next.

4.1 Object Recognition

It is well established that the hippocampus is essential for declarative memory (Cohen & Eichenbaum, 1993; Eichenbaum, 2000). Object recognition tasks have been effective in demonstrating the importance of the hippocampus in both spatial (Zola et al., 2000) and non-spatial memory (Clark et al., 2000; Cohen et al., 2013; Hammond, 2004; Kesner & Novak, 1982).

SK channels are distributed throughout the hippocampus with homomeric SK1channels (in humans) and heteromeric SK1-containing channels (in rodents) robustly expressed in the CA1 and CA3 regions of the hippocampus (Sailer, Kaufmann, Marksteiner, & Knaus, 2004; Stocker et al., 1999). SK1 localization in the plasma membrane and subcellular regions in the soma and the dendrites of pyramidal neurons suggests that they play a role in modulating neuronal excitability and LTP, respectively, in a manner similar to the SK2 and SK3. In order to determine the role of SK1 activation in hippocampal dependent non-spatial memory that enables a clear distinction between memory processes of encoding, consolidation, and retrieval, mice were treated with GW542573X, a highly selective SK1 agonist (Hougaard et al., 2009) and tested in object recognition encoding and consolidation tasks.

Mice treated pre-sample with a systemic injection of the SK1 agonist GW542573X acquired in the sample object exploration criterion in a similar latency as vehicle-treated mice. There also was no significant difference in distance traveled or velocity demonstrated during the sample session. During the test session, pre-sample GW542573X-treated mice exhibited significantly less preference for exploring the novel object than vehicle-treated mice, as determined by discrimination ratio.

These results suggest that activating SK1 impaired object memory encoding. In order to determine whether activating SK1 also affected consolidation, the protocol was repeated using a post-sample treatment of GW542573X or vehicle in place of a pre-sample treatment. During conditioning both controls and mice marked for GW542573X treatment acquired in a similar latency. Post-sample GW542573X-treated mice demonstrated no significant difference in object preference compared to vehicle-treated mice 24 h later, as determined by discrimination ratio.

It is difficult to determine precisely where encoding ends and consolidation begins. However, in the initial study, mice received GW542573X pre-sample and demonstrated impairment. In the following protocol, mice received GW542573X post-sample and did not demonstrate impairment. This suggests that activating SK1 with GW542573X disrupted memory encoding but not memory consolidation.

These results are consistent with Vick et al. (2010) where SK activation by EBIO or CyPPA disrupted encoding of non-spatial object memory. Mice received pre-sample EBIO or CyPPA 20-30 min prior to the sample session. Mice were allowed 10 min exploration time to accumulate 30 s on each object or 38 s on one object. Twenty-four h later, mice that received pre-sample EBIO or CyPPA exhibited significantly less exploration of the novel object than did mice that received pre-sample vehicle.

Stackman et al. (2002) demonstrated that blocking SK channels with apamin increased the number of action potentials (AP) discharged in CA1 neurons of the hippocampus. Also, apamin-treated hippocampal slices given high-frequency stimulation facilitated induction of synaptic plasticity, while low-frequency stimulation did not alter

synaptic strength. These results suggest that apamin shifts the frequency response to lower frequencies and facilitates synaptic plasticity.

Also in Stackman et al. (2002), mice were given pre-sample apamin and then allowed 10 min exploration time to accumulate 19 s per object. Mice that received pre-sample apamin demonstrated significantly greater novel object preference than vehicle-treated mice during a test session 24 h later. Interestingly, when given 38 s to acquire during sample session, there was no significant difference between groups. These results suggest that while apamin enhances weak memory, it does not affect strong memory.

It is unclear why mice demonstrated memory enhancement when SK was blocked by apamin in a weak memory protocol but not a strong memory protocol, and SK1 activated by GW542573X impaired memory in a strong memory protocol. It may be due to the differences in pharmacological compounds, the physiological effect of blocking a channel verses activating a channel, or an inherent difference in the physiology of SK1. Alternately, there may be a difference in hippocampal function in weak versus strong memory, which causes differing influences of SK when activated or blocked. In order to further determine if GW542573X impaired encoding, studies increasing the amount of sample exploration time could be conducted to see if object memory is rescued. If object memory could be rescued, it would suggest an encoding impairment. However, these results do suggest that blocking apamin sensitive SK does enhance memory encoding, while activating SK1 impairs memory encoding in hippocampal-dependent tasks.

There is a robust distribution of SK1 within the hippocampus. The role of the hippocampus in non-spatial object memory has been demonstrated previously. These factors, along with the ability of GW542573X to target SK1 specifically, suggest that

SK1 in the hippocampus is being activated and that activation disrupts the encoding, but not consolidation, of non-spatial object recognition memory. A contextual and cued fear conditioning protocol was next examined to determine if activating SK1 impaired encoding in tasks where it has been demonstrated that the hippocampus is involved but may not necessarily be required.

4.2 Contextual and Cued Fear Conditioning

Pavlovian fear conditioning is used to test contextual and cued fear memory. Contextual fear memory involves the lateral amygdala and the hippocampus, while cued fear memory is dependent on the amygdala (Kim & Fanselow, 1992; Phillips & LeDoux, 1992). McEchron et al. (1998) and Huerta et al. (2000) found that hippocampal involvement was not necessary for conditioned fear responses in contextual and cued fear conditioning.

Heteromeric SK1 channels are distributed robustly in the rodent hippocampus, but with low levels in the basolateral amygdala (Sailer et al., 2004; Stocker et al., 1999). SK2 channels have robust expression in the basolateral amygdala as well as robust expression in the hippocampus. Because the amygdala also is involved in contextual conditioning, along with the hippocampus, it could be predicted that an SK1 activator would result in no impairment in contextual conditioning. To determine if SK1 plays a role modulating learning and memory in tasks where hippocampal and amygdala involvement has been demonstrated, mice were treated with GW542573X and tested in contextual and cued fear conditioning encoding tasks.

During the 5 min context pre-exposure mice marked to receive vehicle and mice marked to receive GW542573X exhibited no significant difference in freezing. Both

GW542573X-treated and vehicle-treated mice demonstrated increased freezing over the course of the conditioning protocol with no significant difference in freezing between groups. During the cued fear test 24 h later, both groups demonstrated increased freezing to tone, but there was no significant difference in % freezing between GW542573X-treated mice and vehicle-treated mice. Also, during contextual testing there was no significant difference demonstrated in % freezing between groups.

These results demonstrate that activation of SK1 by pre-conditioning systemic administration of GW542573X did not lead to impaired memory encoding in mice in either contextual or cued fear memory. This suggests that activating SK1 does not disrupt memory encoding in tasks that are amygdala-dependent or amygdala and hippocampal-dependent.

These results are consistent with Vick et al. (2010) where SK activation by EBIO did not disrupt encoding in contextual or cued fear conditioning. Mice received EBIO 20-30 min prior to a 3 CS/US conditioning session. Twenty-four h later, mice that received pre-conditioning EBIO did not exhibit a significant difference in freezing in either contextual or cued fear conditioning, compared to mice that received pre-conditioning vehicle. This was repeated with a 1 CS/US protocol with similar results.

This differs somewhat from Hammond et al. (2006) where overexpression of SK2 did cause impaired encoding in contextual and cued memory in a 3 CS/US conditioning protocol. Hammond et al. (2006) also recorded EPSPs in CA1 hippocampal slices, demonstrating that blocking overexpression of SK2 with apamin increased EPSPs and that overexpression of SK2 restricts NMDAR activation. Recordings also revealed that overexpression of SK2 weakened the induction of LTP.

Stackman et al. (2008) also demonstrated that overexpressing SK2 channels caused impaired memory encoding in contextual cued fear memory in a 3 CS/US conditioning protocol. This impairment was rescued by increasing the duration of pre-exposure from one 5 min context exposure to three 5 min context exposures, suggesting that the impairment in context fear memory is the result of a disruption in encoding.

These results suggest that memory impairment caused by SK activation using pharmacological agents may not be exhibited in contextual and cued fear memory because the amygdala may be compensating for the impairment of the hippocampus. However, the amygdala may not be able to compensate for the hippocampus when memory impairment is caused by overexpression of SK2 channels as the amygdala also may be compromised.

When Vick et al. (2010) tested encoding by blocking SK using a pre-conditioning treatment of apamin, there was no significant difference observed in freezing between groups in 3 CS/US contextual or cued fear conditioning. Interestingly, however, in a 1 CS/US protocol, there was a significant increase in freezing in mice that received pre-conditioning apamin compared to mice that received pre-conditioning vehicle during contextual but not tone tests.

The results in Vick et al. (2010) correspond to the results in Stackman et al. (2002) where weak memory in novel object recognition encoding was enhanced by blocking apamin sensitive SK, but strong memory was not. This difference in results between the use of SK blockers rather than SK1 activators remains consistent, suggesting that while apamin may enhance memory encoding by reducing the amount of training needed to acquire lasting memory in hippocampal-dependent spatial and object memory,

the impairment observed after SK activators may be overcome by increasing the amount of training needed. Future study is necessary to determine the physiological mechanisms behind these differing results.

There is robust distribution of SK1 within the hippocampus with only low levels expressed in the amygdala. The hippocampus along with the amygdala has been demonstrated to be involved in contextual fear, while the amygdala has been demonstrated to be involved in cued fear. The demonstrated ability of GW542573X to target SK1 specifically suggests that SK1 in the hippocampus is being activated; however, that activation does not disrupt encoding in contextual or cued fear conditioning. A post-sample protocol was not pursued due to the lack of results demonstrated in the encoding protocol. A trace fear protocol was examined next to determine if activation of SK1 would impair encoding and/or consolidation in hippocampal-dependent temporal tasks.

4.3 Trace Fear Conditioning

It is established that in humans the hippocampus is required for episodic memory, both spatial and temporal (Cohen & Eichenbaum, 1993). In rodents, NMDA receptor (NMDAR) mediated plasticity in the CA1 region of the hippocampus is essential for spatial (O'Keefe, 1999) and temporal memory (Huerta et al., 2000). The hippocampus is required for encoding the duration of the trace interval between the CS and the US in trace fear conditioning (McEchron et al., 1998; McEchron, Tseng, & Disterhoft, 2003).

McEchron et al. (2003) demonstrated that CA1 hippocampal neurons fired during the trace intervals in trace fear tone tests. Rabbits received one of two conditioning protocols consisting of 35 CS/US pairings and either a 10 s or a 20 s trace separating the

tone (CS), followed by a 150 ms airpuff to the cornea (US). Recordings from pyramidal neurons of the CA1 during a tone test 24 h later indicated maximal firing of action potentials in neurons at 10 s after the trace, if given the 10 s trace protocol, and at 20 s after the trace, if given the 20s trace protocol. These results demonstrate the necessary involvement of CA1 hippocampal neurons during trace fear conditioning.

Using NMDAR knockout mice in trace fear and delay fear protocols, Huerta et al. (2000) found that NMDARs in the CA1 region of the hippocampus were required for the formation of memories across time. NMDAR knockout mice were given a 10 CS/US paired protocol with a 30 s trace interval between the tone (CS) and the footshock (US). During conditioning, NMDAR knockout mice demonstrated significantly less freezing than controls after the first inter-trial interval (ITI); but freezing was not significantly different by the end of the training session, possibly as a result of habituation. During the tone test session, NMDAR knockout mice exhibited significantly less freezing than controls. There was no significant difference demonstrated between groups during the contextual fear test. Additionally, Huerta et al. (2000) compared these results to results obtained in a 3 CS/US fear conditioning protocol, where the hippocampus is involved but not required (McEchron et al., 1998), and found no significant difference between NMDAR knockout mice and controls in either contextual or cued fear conditioning.

The robust distribution of SK1 throughout the hippocampus suggests a role in the modulation of NMDAR mediated plasticity in temporal memory. In order to determine the role of SK1 activation in hippocampal dependent temporal memory, mice were treated with the SK1 agonist GW542573X and tested in trace fear conditioning.

During the conditioning session there was no significant difference in activity burst between pre-conditioning GW542573X and vehicle-treated mice. While both GW542573X-treated and vehicle-treated groups demonstrated increased freezing over the course of the trace fear conditioning protocol, there was no significant difference in freezing between groups. GW542573X-treated mice exhibited numerically less freezing than vehicle-treated mice, but not significantly. These results were consistent with the results in Huerta et al. (2000). GW542573X-treated mice, similar to NMDAR knockout mice, demonstrated less freezing than vehicle-treated mice in earlier ITI, with less freezing between groups by the end of the training session. Both GW542573X-treated mice in the present study and NMDAR knockout mice in Huerta et al. (2002) were able to learn the task, but were slower in acquiring the task than the controls.

During the tone test 24 h later, both GW542573X-treated mice and vehicle-treated mice demonstrated increased freezing to tone with a significant difference in % freezing between groups. GW542573X-treated mice froze significantly less in the 15 s trace period following the tone than did vehicle-treated mice. % freezing dropped in both groups in the second 15 s trace period corresponding with the second post-CS time bin. Consistent with the results found during the context test in the contextual and cued fear conditioning protocol, there was no significant difference demonstrated between groups during the context test in the trace fear conditioning protocol.

The results demonstrate that activation of SK1 by pre-conditioning systemic administration of GW542573X led to impaired memory encoding in mice in trace fear memory but not in contextual trace fear tests. This suggests that activation of SK1 affected memory in tasks that were selectively dependent upon the hippocampus.

In order to determine whether activating SK1 also affected consolidation, the protocol was repeated using a post-conditioning treatment of GW542573X or vehicle in place of a pre-conditioning treatment. While both groups demonstrated increased freezing over the course of the trace fear conditioning protocol, there was no significant difference in freezing between groups.

During the tone test 24 h later, both post-conditioning GW542573X-treated mice and vehicle-treated mice demonstrated increased freezing with no significant difference in % freezing between groups. There was no significant difference in % freezing during the 15 s trace period following the tone between GW542573X-treated and vehicle-treated mice. % freezing dropped in both groups in the second 15 s trace period corresponding with the time bin post-shock. Also, no significant difference was demonstrated in % freezing between groups during the context test.

The initial study, where mice received GW542573X pre-conditioning and demonstrated impairment, was followed with a protocol in which mice received GW542573X post-conditioning and did not demonstrate impairment. These results suggest that activating SK1 with GW542573X disrupted memory encoding, but not consolidation in trace fear conditioning. No significant memory impairment was exhibited during contextual trace fear conditioning.

The results demonstrated in the pre-conditioning and post-conditioning contextual and trace fear conditioning protocols are consistent with those obtained in McKay et al. (2012). McKay et al. used NS309, an SK2 channel activator, to determine its effect on apamin sensitive afterhyperpolarization (AHP) in the CA1 of the hippocampus during hippocampal-dependent trace eye-blink conditioning in rats. Rats received pre-

conditioning NS309 followed by a 30 CS/US paired conditioning protocol with a 250 ms trace separating a 250 ms tone (CS) from a 100 ms airpuff to the cornea (US). NS309-treated rats were significantly impaired in acquisition of eyeblink conditioning compared to vehicle-treated rats. Both NS309-treated mice in McKay et al. (2012) and GW542573X-treated mice in the present study acquired at a slower rate and at lower level than controls.

McKay et al. (2012) also examined the reduction of excitability in CA1 hippocampal neurons by NS309 in vitro. A bath application of NS309 significantly increased the peak of the medium post-burst afterhyperpolarization (AHP) with no effect on the slow AHP and reduced EPSP by increasing Ca^{2+}-dependent outward currents near the synapse. Molecular assays also were conducted to verify changes in SK2 channels in hippocampal pyramidal neurons as a result of learning. Significant reductions in SK2 gene mRNA levels and SK2 protein levels in eyeblink conditioned rats were discovered. These results confirm the finding in McEchron et al. (2003) that the hippocampus is required in order to associate the CS with the US over a trace period.

In the current study, mice treated pre-conditioning with GW542573X were unable to form an association between the CS and the US across the trace interval as opposed to the vehicle-treated mice. There is a robust distribution of SK1 within the hippocampus. The hippocampus has been demonstrated to be required in trace fear conditioning. These factors, along with the demonstrated ability of GW542573X to target SK1 specifically, suggest that SK1 in the hippocampus are being activated and that activation disrupts encoding in trace fear conditioning. Both the hippocampus and the amygdala are involved in contextual fear conditioning, suggesting that even if GW542573X activates

SK1 expressed in the hippocampus, that activation may not disrupt encoding in contextual conditioning, possibly as a result of compensatory processes in the amygdala.

Temporal memory has been demonstrated to be hippocampal dependent (McEchron et al., 1998; McEchron et al., 2003). The current study is consistent with results demonstrated in previous studies on the activation of SK2 and SK3. Here we see clear evidence that pre-conditioning activation of SK1 in hippocampal-dependent temporal memory tasks impairs encoding. Pre-conditioning activation of SK1 in hippocampal and amygdala-dependent contextual memory and amygdala-dependent cued fear did not impair memory encoding. The trace fear conditioning protocol does not lead to strong context conditioning due to lack of pre-context exposure. When a 3CU/US contextual and cued fear conditioning protocol was used there was no impairment in contextual conditioning. GW542573X was not administered until after context memory encoding during the pre-exposure session. As a result of this, the association of the context memory with the US was not affected. The results of the current study suggest that SK1 plays a similar, but independent role in learning and memory from other members of the SK channel family.

4.4 Different Roles for SK Subunits

Unlike EBIO and CyPPA, which facilitate activation of SK by increasing Ca^{2+} sensitivity (Vick et al., 2010), GW542573X activation of SK1 does not depend on the CaMBD in the C terminus for activation as do the SK2 and SK3, but rather by a "deep pore" gating structure (Ser 293) in the inner pore of transmembrane segment 5 (Hougaard et al., 2009). This activation by means other than Ca^{2+} suggests a possible indication that SK1 may be activated in situations where Ca^{2+} supplies are too low for the activation of

SK2 and SK3. This may provide a backup system for activating heteromeric SK2 channels and homomeric SK1 channels in humans, allowing the regulation of mAHPs and constraining intrinsic excitability when necessary. Future research on the independent workings of SK1 will clarify its role as a subunit of heteromeric SK2 and SK3 channels and as a homomeric channel.

The fact that SK1 homomeric channels are selectively co-localized with D class L-type channels, long lasting voltage dependent Ca^{2+} channels (Bowden et al., 2001; Stocker, 2004), rather than being expressed in close proximity to NMDAR as are SK2 channels, also plays a role in this delicate balance of Ca^{2+} activation. The activation of SK1 channels by Ser 293, along with the co-localization D class L-type Ca^{2+} channels, suggests differing physiological roles in synaptic transmission, plasticity, and memory encoding for SK1 channels independent of SK2 and SK3 channels.

Ca^{2+}-independent interactions with CaM are required for surface expression of SK channels (Adelman et al., 2012; Lee et al., 2003; Stocker, 2004). The multiple phosphorylation sites, which increase Ca^{2+} sensitivity, found on SK channels modulate SK channel assembly and LTP dependent trafficking in CA1 pyramidal neurons (Adelman et al., 2012). Subcellular localization of SK1 is found in rodent CA1 hippocampal neurons (Bowden et al., 2001), but is only expressed in the plasma membrane when co- expressed with SK2 or SK3 (Stocker, 2004). This suggests that SK1 may regulate the physiological properties of assembly and trafficking of heteromeric and homomeric SK channels in rodents and humans, causing differences in NMDA receptor mediated plasticity, modulating LTP, and affecting memory encoding. Future studies of SK1 and their binding with specific interacting proteins to influence subcellular

localization, assembly, and trafficking would be helpful in further understanding the underlying mechanisms of these SK channel subunits and their involvement in learning and memory as well as multiple disorders including age related memory disorders, depression, and addiction.

V. CONCLUSION

In this study, the effect of a new SK1 selective activator, GW542573X (Hougaard et al., 2009), on hippocampal dependent object recognition memory, contextual and cued fear conditioning, and on contextual and tone trace fear conditioning in male C57BL6J mice was examined. The results suggest that activation of SK1 plays a significant role, independent of SK2 and SK3, in memory encoding.

The present study suggests that activation of SK1 modulates afterhyperpolarization of post-synaptic CA1 hippocampal neurons by causing an efflux of excess K^+, which decreases the number of action potentials fired by the neurons, reducing excitability. This hyperpolarization of the post-synaptic membrane may impair memory by suppressing NMDAR-mediated plasticity. The current data supports a role for SK1 in the modulation of hippocampal synaptic plasticity and hippocampal-dependent memory.

APPENDICES

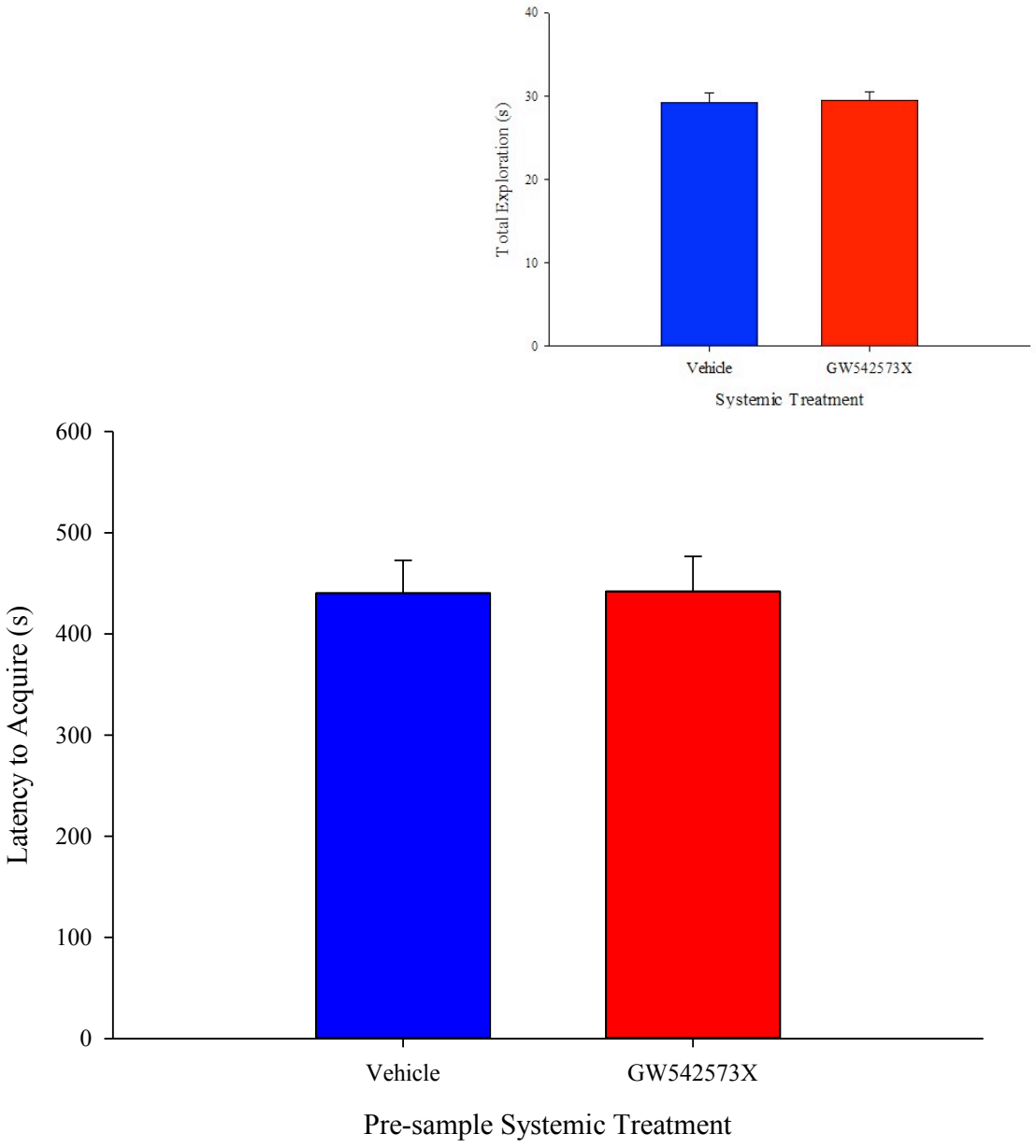


Figure 1.A. Object Recognition Test Session Pre-sample GW542573X. Mice treated with GW542573X, $n = 11$, acquired the sample object exploration criterion in a similar latency as vehicle-treated mice, $n = 13$, $t(24) = -.04$, *ns*, mean (± S.E.M.). *Inset.* Total exploration time between groups was not significantly different between treatment conditions, $t(24) = 158$, *ns*, mean (± S.E.M.).

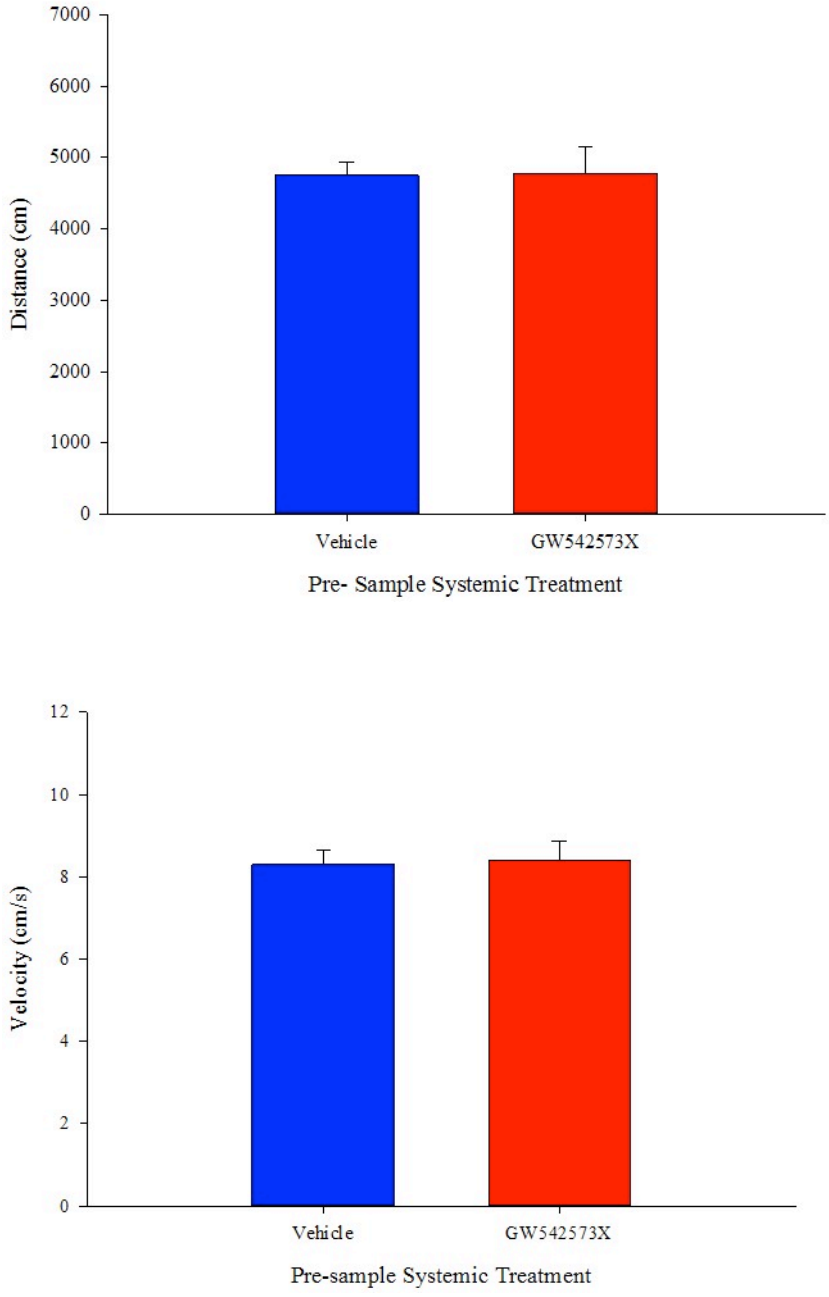


Figure 1.B. Object Recognition Sample Session Pre-sample GW542573X Distance Traveled and Velocity. Distance traveled during the sample session was not significantly different between the vehicle-treated group and the GW542573X-treated group, $t(24) = .387$, *ns*. Velocity between groups was also non-significant between groups, $t(24) = 0.23$, *ns*, mean (± S.E.M.).

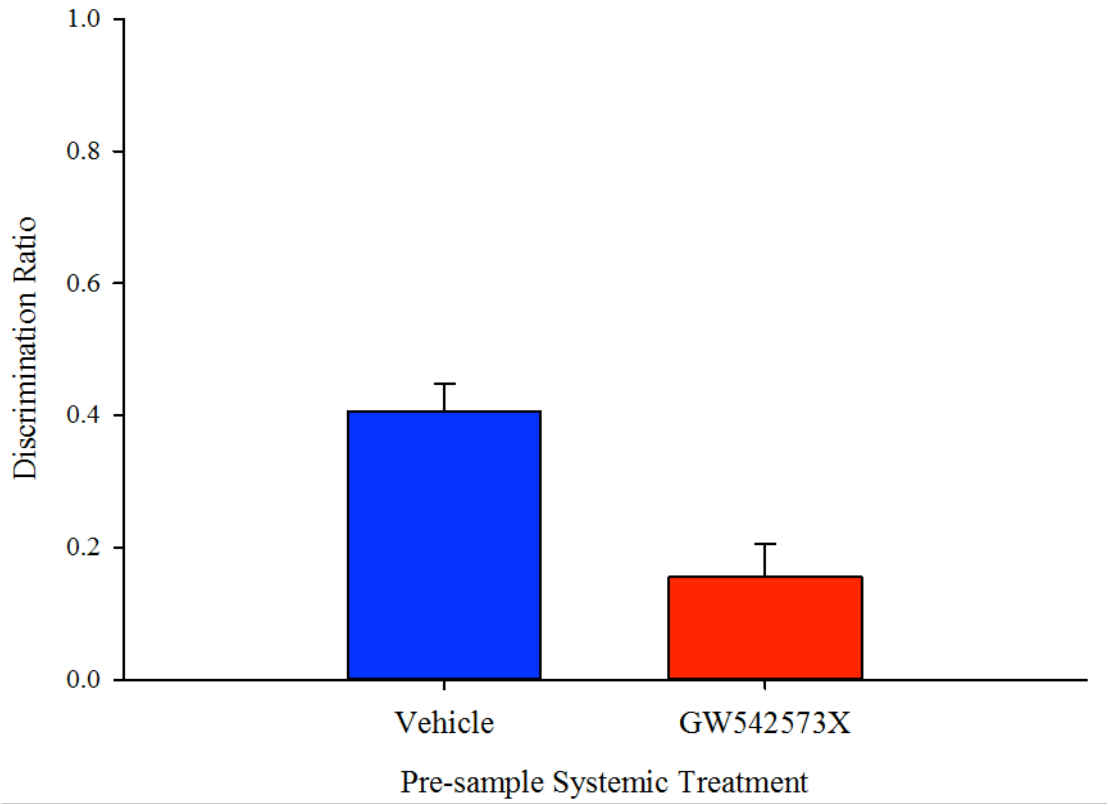


Figure 2. Object Recognition Test Session Pre-sample GW542573X. Pre-sample vehicle-treated mice, $n = 13$, exhibited a significant novel object preference, as inferred from the discrimination ratio, in contrast to mice that had received pre-sample GW542573X, $n = 11$, $t(24) = 3.88$, $p = 0.009$, mean (± S.E.M.).

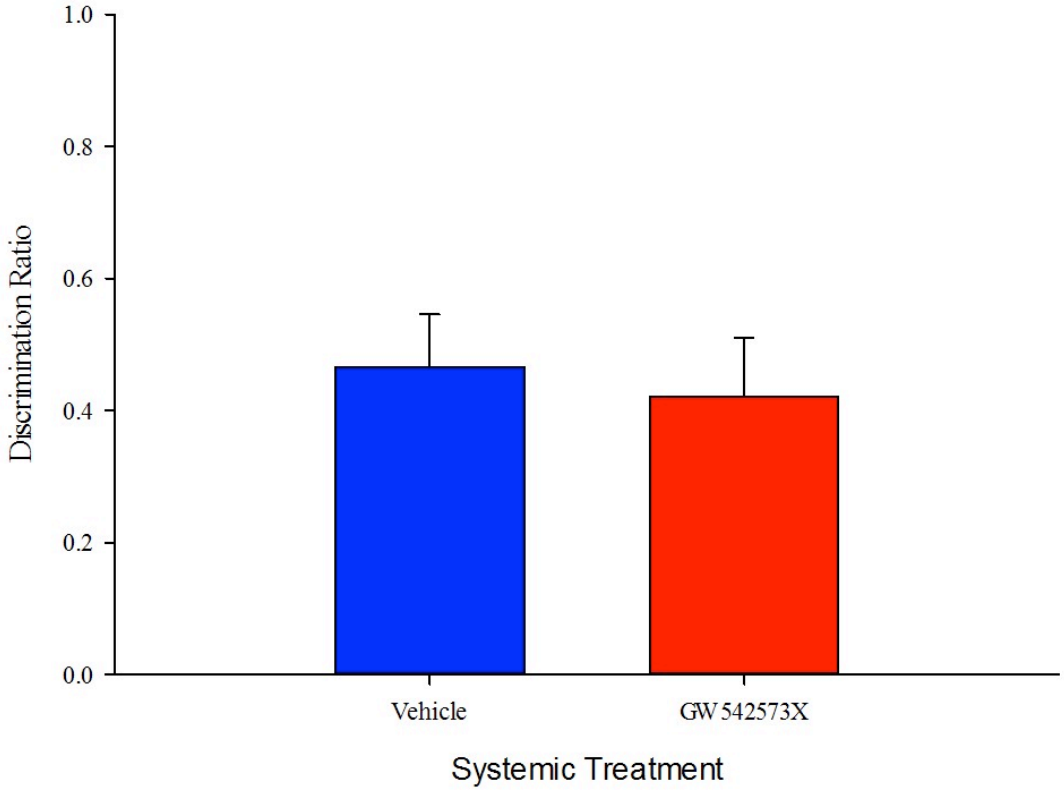


Figure 3. Object Recognition Post-sample GW542573X. Mice that received post-sample vehicle, $n = 9$, and GW542573X-treated mice, $n = 8$, displayed similar test session discrimination, as inferred from the discrimination ratio, $t(15) = 0.38$, *ns*, mean (± S.E.M.).

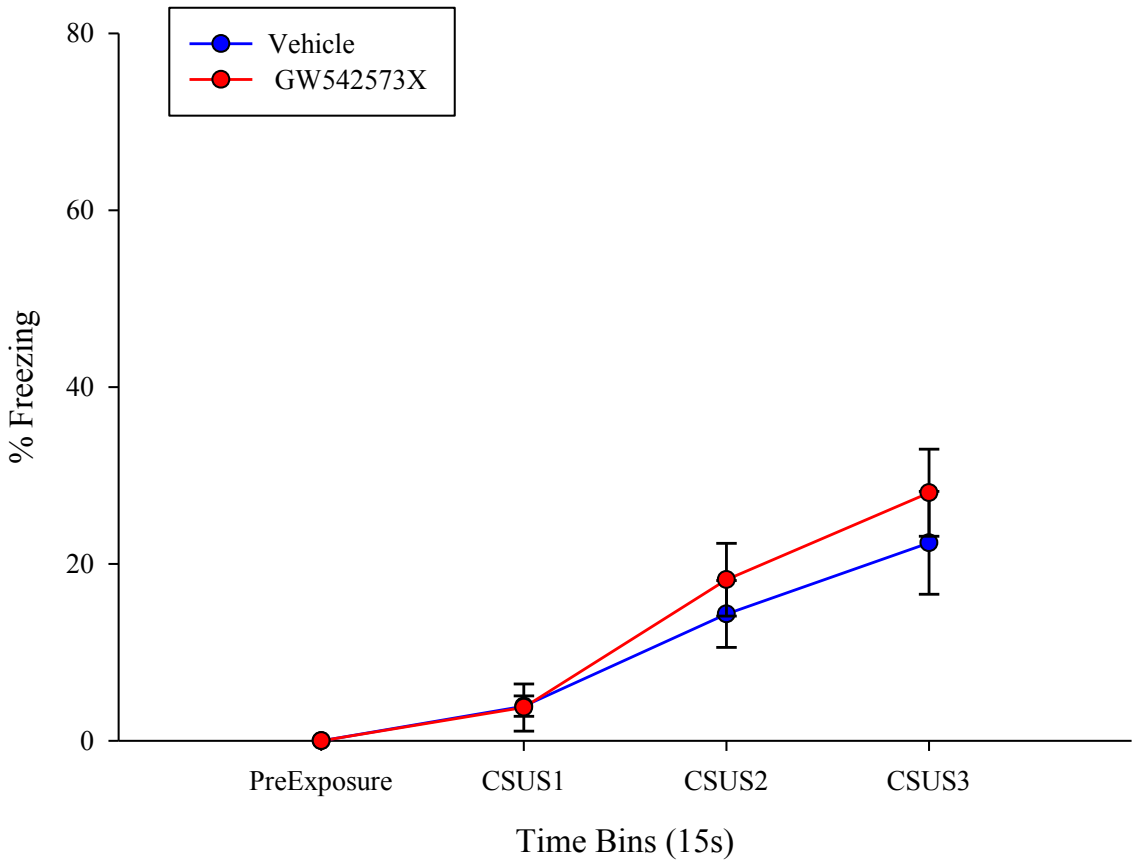


Figure 4.A. Cued Fear Conditioning Pre-conditioning GW542573X. Mean (± S.E.M.) shows increased freezing during conditioning by both groups, but no significant difference between the vehicle-treated group, $n = 6$, and the GW542573X-treated group, $n = 6$, $F(1,10) = 0.47$, *ns*.

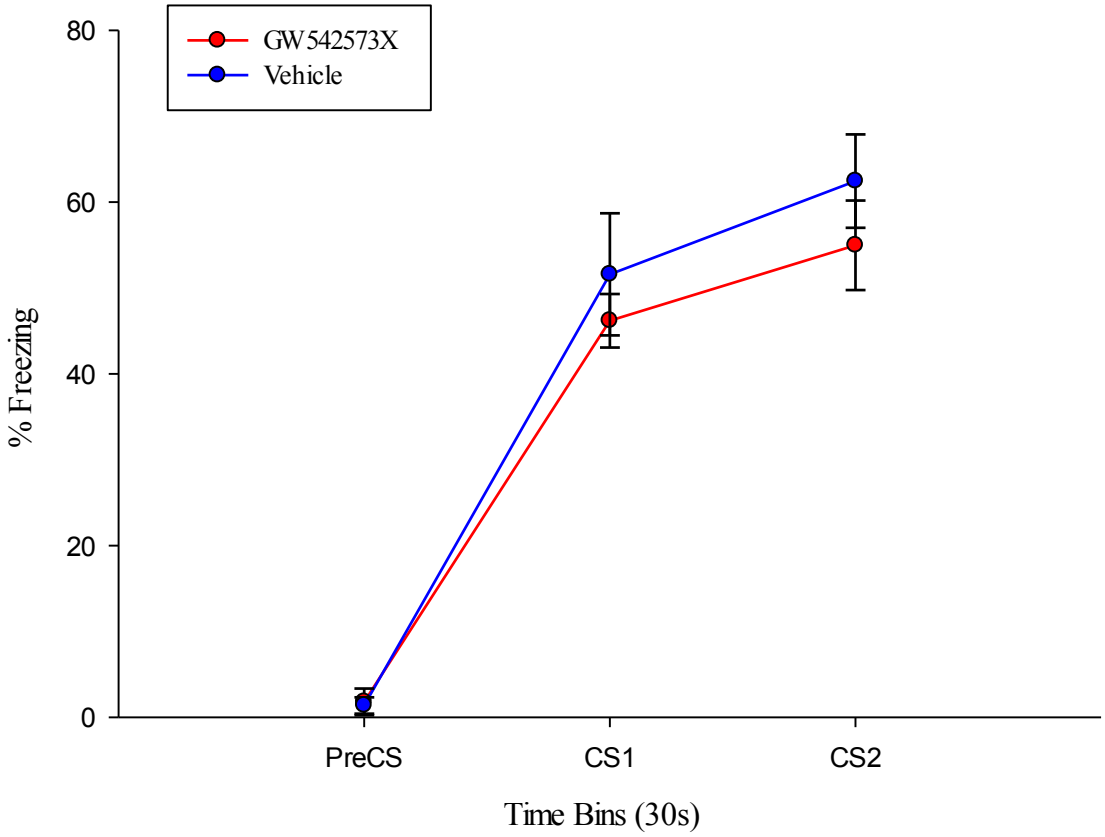


Figure 4.B. Cued Fear Memory Test Pre-conditioning GW542573X. There was no significant effect of treatment between groups exhibited during cued fear memory test, GW542573X-treated, $n = 6$, or vehicle-treated, $n = 6$, $F(1,10) = 0.72$, *ns*, mean (± S.E.M.).

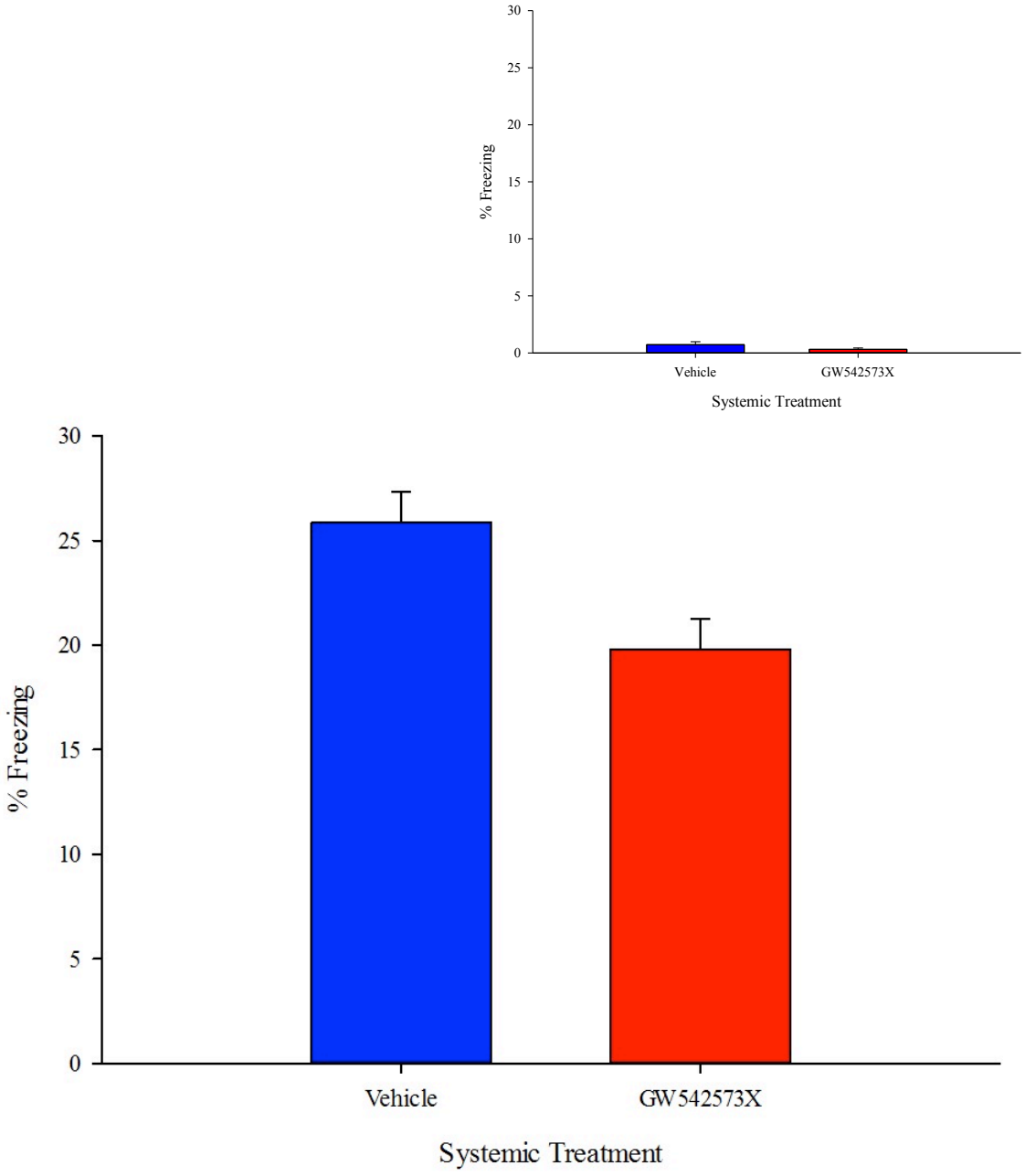


Figure 4.C. Contextual Memory Test Pre-conditioning GW542573X. Mean (± S.E.M.) total % freezing during the context test 24 h after conditioning, was not significantly different between mice that received pre-conditioning vehicle, $n = 6$, and GW542573X, $n = 6$, $t(10) = 0.74$, *ns*. *Inset.* Context Pre-exposure. There was no significant difference in % freezing during pre-exposure between mice marked for vehicle-treatment, $n = 6$, and mice marked for GW542573X-treatment, $n = 6$, $t(10) = 1.33$, *ns*, mean (± S.E.M.).

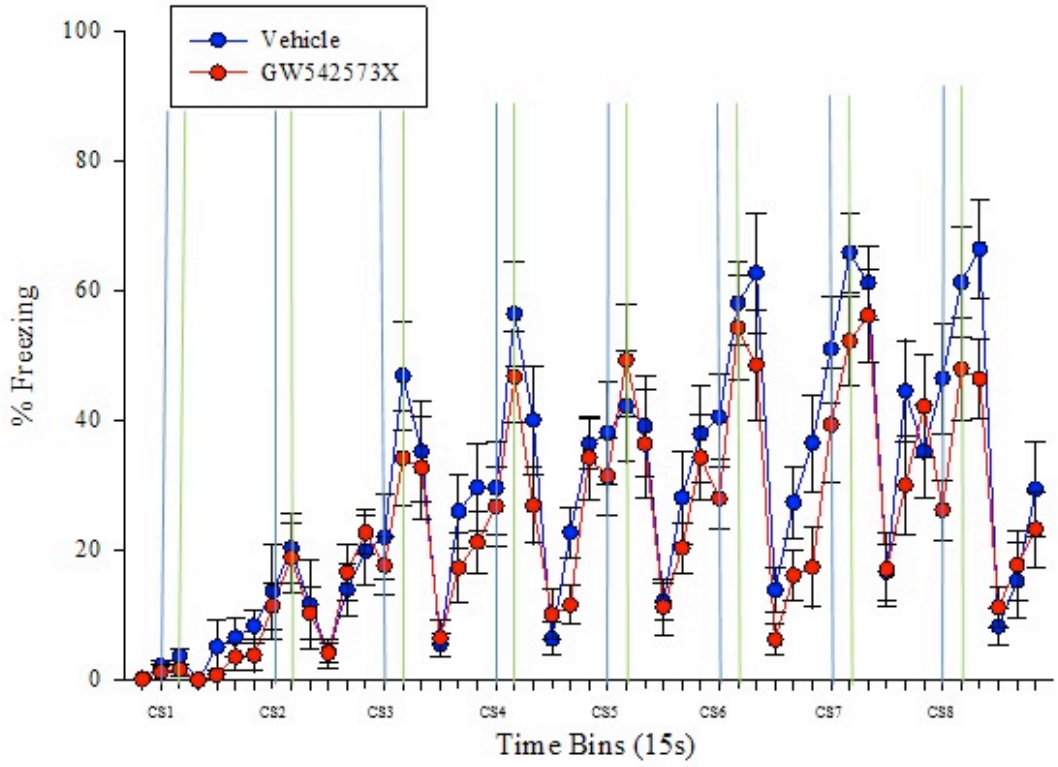
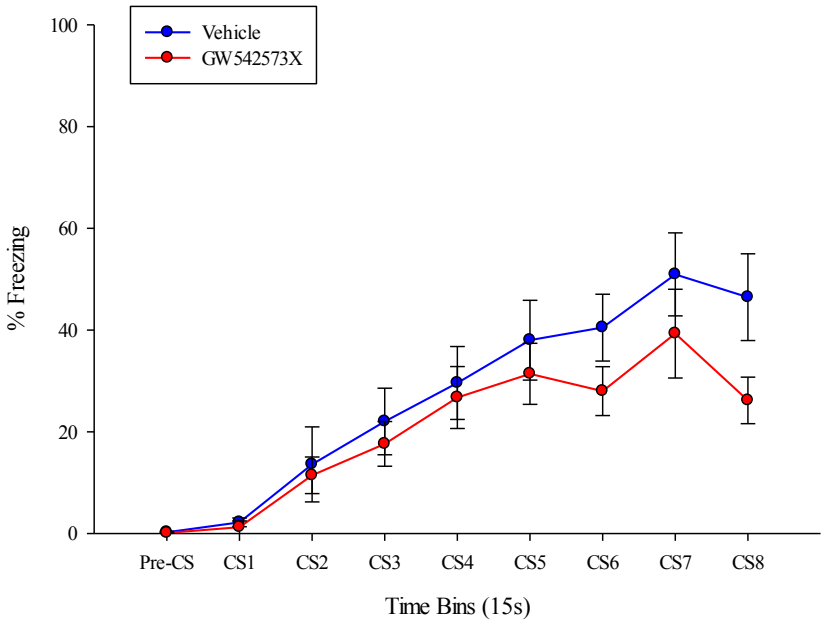


Figure 5.A. Trace Fear Conditioning Pre-conditioning GW542573X. Mice that received pre-conditioning GW 542573X (15 mg/kg), $n = 11$, exhibited less freezing during each CS/US pairing compared to vehicle-treated mice, $n = 13$. There was no significant overall treatment effect between groups, $F(1,22) = 2.23$, *ns*, mean (± S.E.M.). (Blue = CS, Green = US)



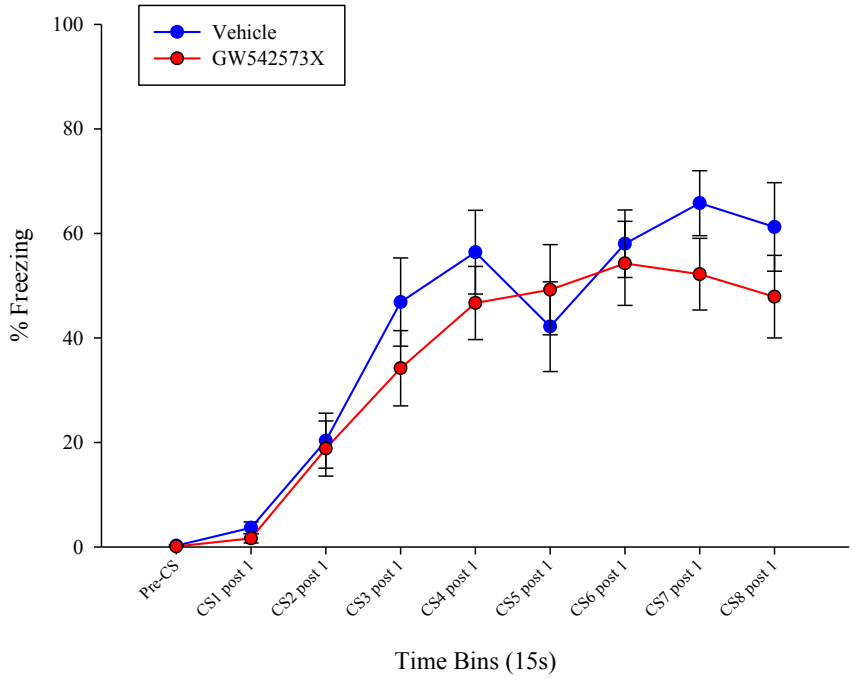


Figure 5.B. Trace Fear Conditioning CS Pre-conditioning GW542573X (top) and Trace Fear Conditioning CS Post 1 Pre-conditioning GW542573X (bottom). Mice that received pre-conditioning GW 542573X (15 mg/kg), $n = 11$, exhibited less freezing during each CS, and during each CS post-1, compared to vehicle-treated mice, $n = 13$. There was no significant overall treatment effect exhibited between groups, CS, $F(1,22) = 2.38$, *ns*, CS post 1, $F(1, 22) = 1.81$, *ns*, mean (± S.E.M.).

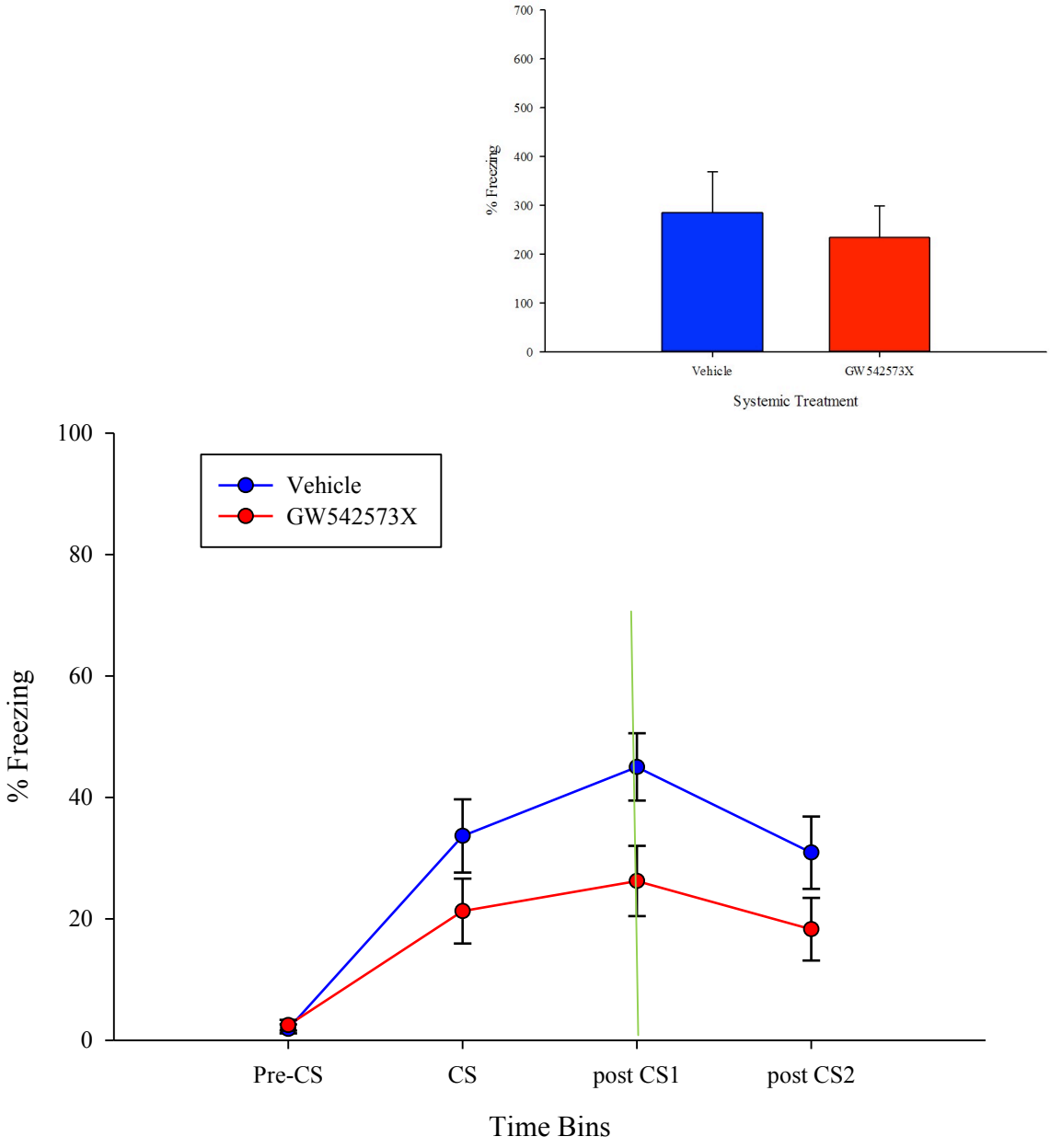


Figure 5.C. Trace Fear Memory Test Pre-conditioning GW542573X. GW542573X-treated mice, $n = 11$, exhibited significantly less freezing during trace tone test compared to vehicle-treated mice, $n = 13$, $F(1,22) = 5.17$, $p = 0.03$, mean (± S.E.M.). (Green line represents where US was received during conditioning session.) *Inset.* Activity Burst. Total % freezing, mean (± S.E.M.), during activity burst pre CS and post-CS was not significantly different between the mice that received pre-conditioning vehicle or GW542573X.

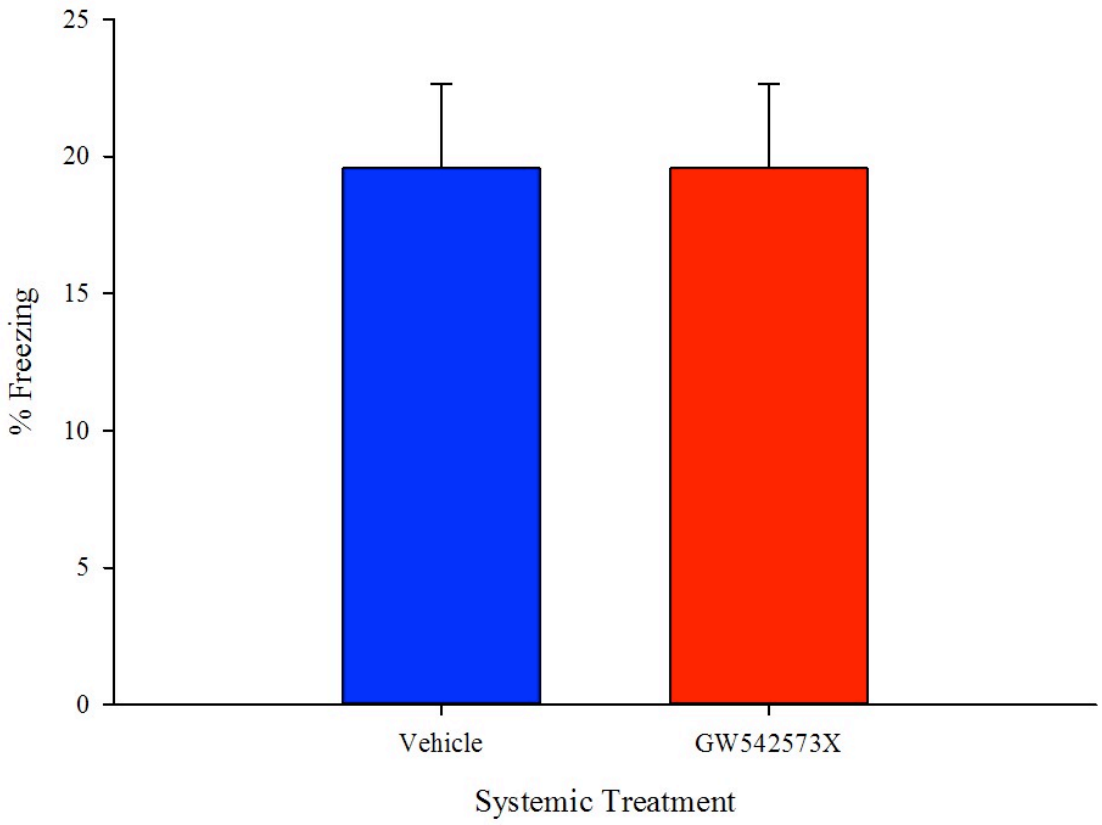


Figure 5.D. Trace Fear Memory Context Test Pre-conditioning GW542573X. Mean (± S.E.M.) total % freezing during the context test 24 h after conditioning was not significantly different between the mice that received pre-conditioning vehicle, n = 13, and GW542573X-treated mice, n = 11, $t(9) = -0.86$, *ns*.

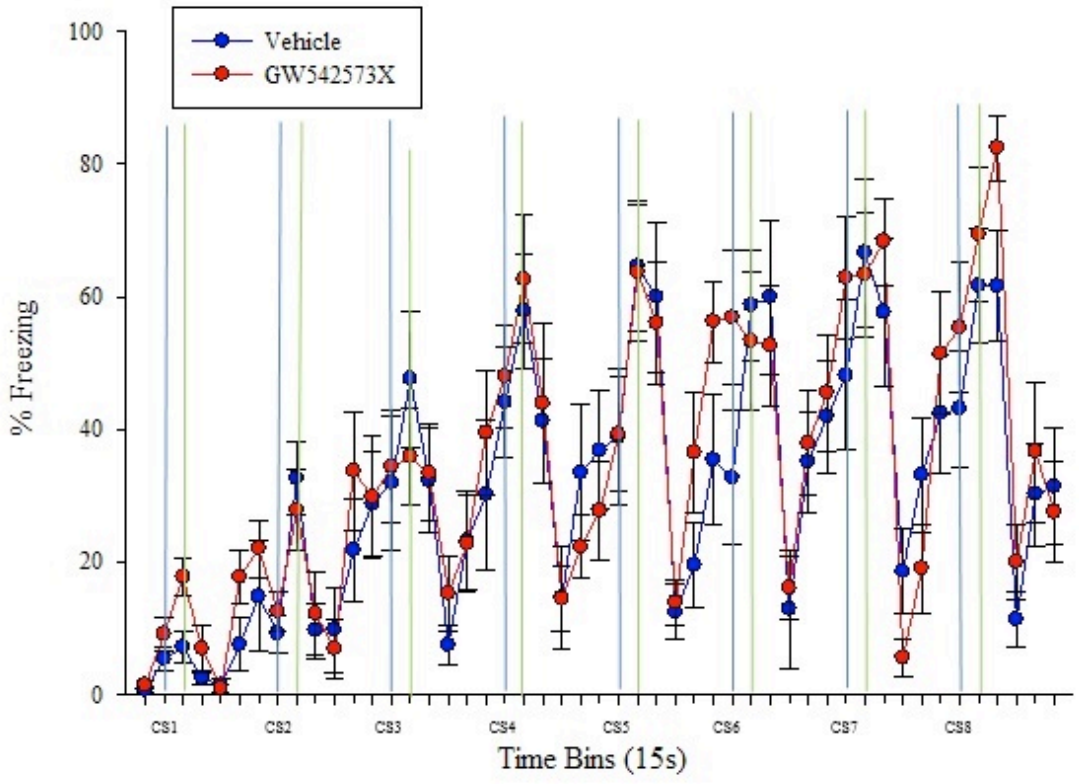
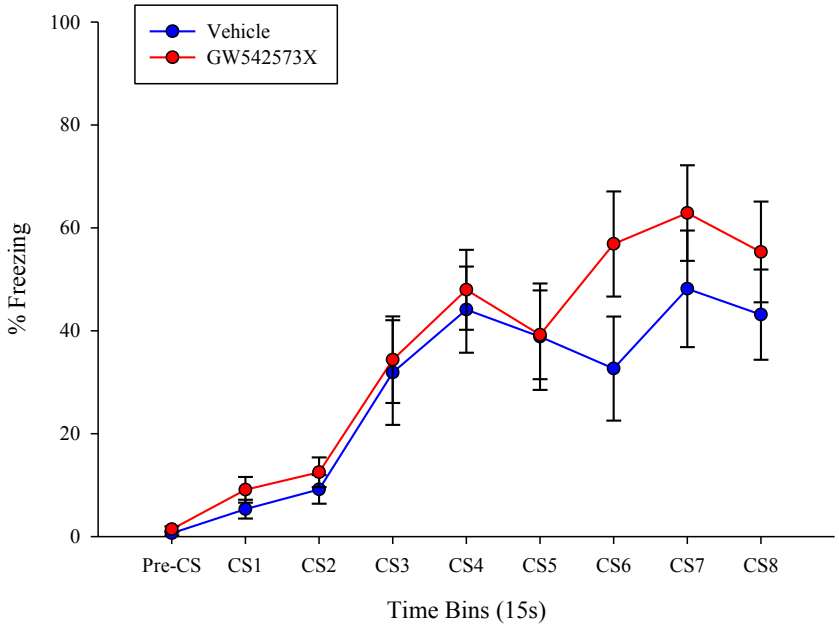


Figure 6.A. Trace Fear Conditioning Post-conditioning GW542573X. Freezing between mice marked to receive post-conditioning GW 542573X (15 mg/kg), n = 9, was not significantly different than mice marked to receive post-conditioning vehicle, n = 9, $F(1,16) = 0.28$, *ns*, mean (± S.E.M.). (Blue = CS, Green = US)



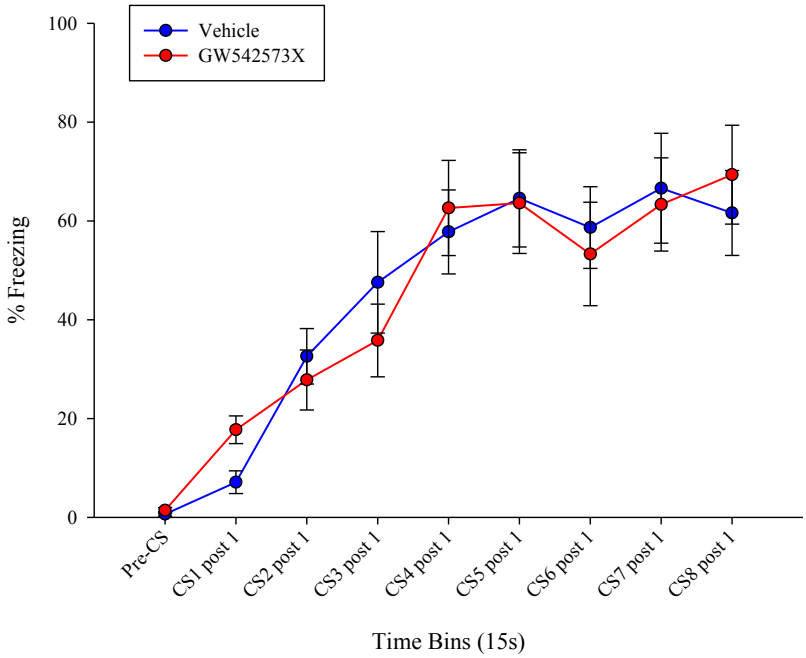


Figure 6.B. Trace Fear Conditioning CS Post-conditioning GW542573X (top) and Trace Fear Conditioning CS Post 1 Post-conditioning GW542573X (bottom). Freezing between groups at the CS interval, $F(1, 16)= 1.14$, *ns*, and the CS post 1 interval, $F(1,16) = 0.06$, *ns*, was not significantly different between mice marked to receive post-conditioning GW 542573X (15 mg/kg), n = 9, and mice marked to receive post-conditioning vehicle, n = 9, mean (± S.E.M.).

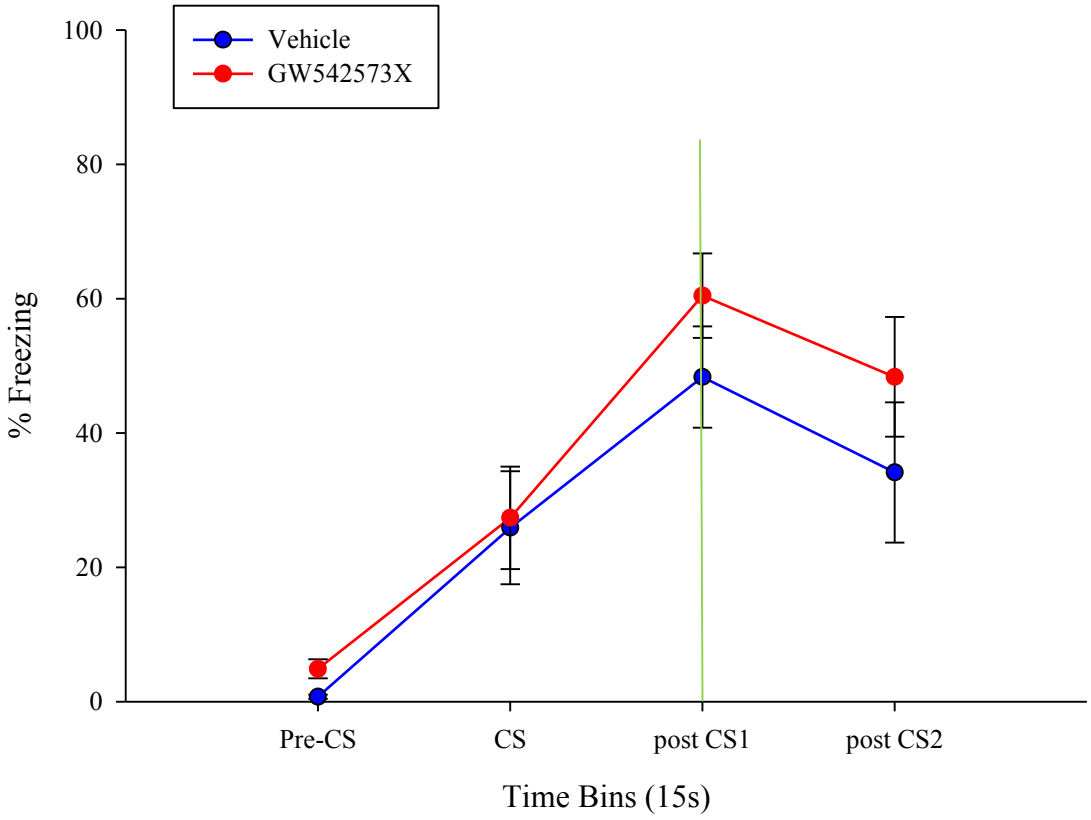


Figure 6.C. Trace Fear Memory Test Post-conditioning GW542573X. GW542573X-treated, $n = 9$, mice did not exhibit significantly less freezing during trace tone test compared to vehicle-treated mice, $n = 9$, $F(1,16) = 1.24$, *ns*, mean (± S.E.M.). (Green line represents where US was received during conditioning session.)

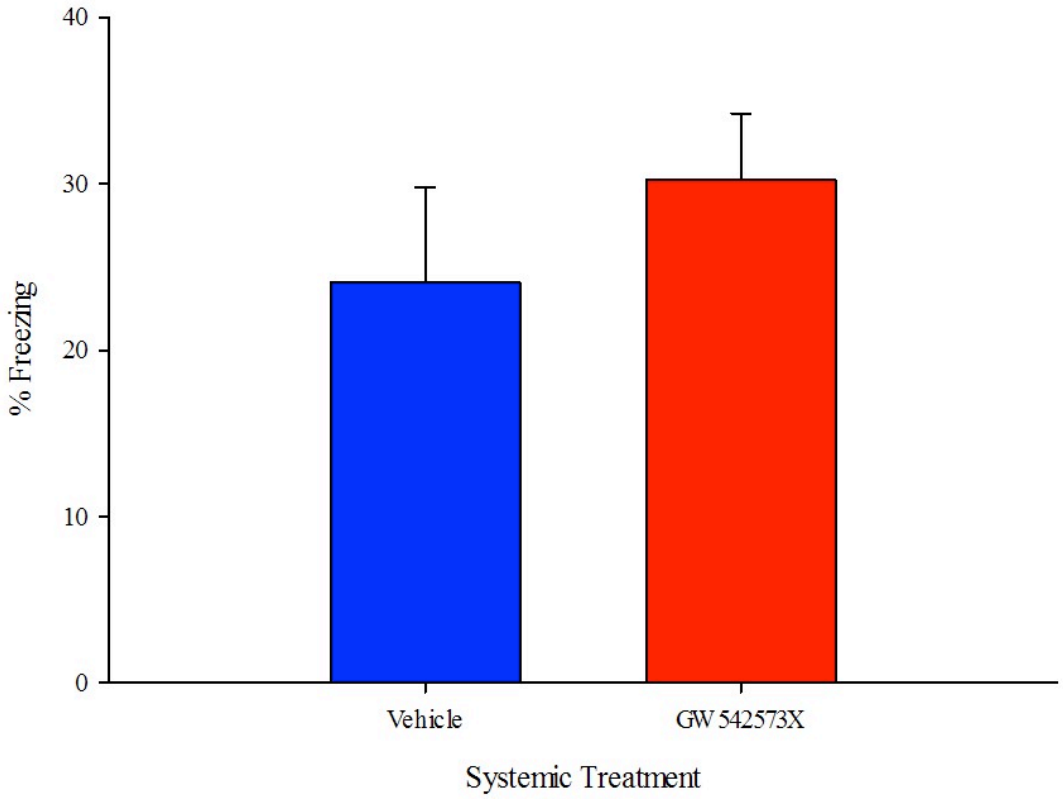


Figure 6.D. Trace Fear Memory Context Test Post-conditioning GW542573X. Mean (± S.E.M.) total % freezing during the context test 24 h after conditioning was not significantly different between the mice that received post-conditioning vehicle, n = 9, and post-conditioning GW542573X, n = 9, $t(16) = 0.88$, *ns*.

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