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Role of p38 MAPK inhibitor in conditioned fear

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ABSTRACT

p38 mitogen activated protein kinase (p38) is a kinase that has been implicated in cellular plasticity, stress, and psychiatric disorders and recently in the process of DNA repair. Recently, we have shown that p38 is responsible for inhibiting Glycogen Synthase Kinase 3β (GSK3β), which has also been shown to be involved in the same processes and recently in the process of DNA repair. We have also shown that GSK3β is regulated by stress and that its inhibition produces exaggerated conditioned fear. The goal of this study is to examine whether inhibiting p38 will result in a similar exaggeration of conditioned fear. To this end, mice were injected systemically with the potent and selective inhibitor of p38, SB203580 or vehicle prior to tone and footshock fear conditioning and tested for freezing to the tone one day later. Mice injected with SB203580 showed greater tone freezing than mice injected with vehicle. In contrast to tone freezing, SB203580 injections did not affect freezing to the context. Injections of SB203580 prior to 24 hours after fear conditioning, but before fear testing, also did not affect freezing. These data suggest that p38 plays a role in regulating the strength of conditioned fear. The fact that p38 regulates GSK3β and that inhibition of GSK3β also produces exaggerated conditioned fear raises the possibility that a p38 to GSK3β pathway may be regulating the strength of conditioned fear.

INTRODUCTION:

Role of p38 MAPK inhibitor in conditioned fear

The mitogen-activated protein kinase (MAPK) pathways mediate cellular signal transduction in response to external stimuli. The four major subgroups of the MAPKs are the extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK/big MAP kinase 1 (BMK1), and the p38 group (Zarubin 2005). The JNK and p38 MAPK families are both referred to as stress-activated protein kinases (SAPK). The p38 MAPKs are sensitive to extracellular stresses and inflammatory cytokines (Cuenda 2007). The p38 MAPKs are activated by dual phosphorylation of tyrosine and threonine sequences and inactivated by dephosphorylation by protein phosphatases (Cuenda 2007). In response to extracellular stimuli, the MAPKs are responsible for cellular cross talk with substrates that lead to diverse functions in the cell (Cuadrado et al., 2010). At least half of the downstream substrates for p38 MAPKs are transcription factors, suggesting p38 MAPKs strong role in gene regulation at the transcriptional level.

p38 is known as a therapeutic target for its involvement in a variety of pathological conditions. p38 activation has been linked to inhibitory roles in the cell cycle and in tumorigenesis (Zarubin 2005). p38 was shown to mediate a protective enzyme in dopaminergic neurons, a cell type impacted by Parkinson's disease (Jiang et al. 2014). p38 and some of its upstream activators have been linked to senescence in response to telomere shortening. Some reports have shown lower p38 activation in tumors, suggesting a regulatory role of p38 over cell division (Zarubin 2005). Inhibition of GSK3-β by p38, results in activation of β-catenin, a protein implicated in cancer (Thornton et al., 2008). The overexpression of β-catenin and subsequent cancers may be linked to its upstream regulators such as GSK3-β and p38. Other

research has found the disruption of p38's upstream regulators as well as direct inactivation of p38 induces tumor growth in rats (Brancho et al., 2003). These findings suggest p38 as a therapeutic target against Parkinson's disease and potentially oncogenic cells.

p38 activation is also necessary for cytokine biosynthesis in a variety of cell types (Lee et al., 2000). These cytokines act as cell signaling proteins between the immune system and the CNS to promote inflammation, tumorigenesis, apoptosis and other functions. p38 is known to be important in cell differentiation associated with regulating both the innate and adaptive immune response (Langosch et al., 2015). Indeed, p38 MAPKs are integral to immune-mediated diseases such as tuberculosis and arthritis. For example, the bacterium behind tuberculosis was found to destabilize the innate immune response by way of the ERK and p38 pathways (Zhou et al., 2010). Research suggests that p38 is also active in lymphocytes in response to disorders such as rheumatoid arthritis (Mavropoulos et al., 2013). SB203580, a potent and selective inhibitor of p38 α and p38 β, emerged as a potential therapeutic agent as it suppressed pro-inflammatory cytokine productions and reduced the severity in the mouse model of arthritis (Badger et al., 1996).

p38 has been implicated in the behavioral and physiological responses to stress. As mentioned earlier, p38 is considered a stress-activated protein kinase (SAPK). Cytokines have been shown to increase in number in times of psychological stress. Specifically, the p38 mediated cytokines, TNF- and IL- are known to regulate the serotonin transporter protein in response to antidepressants (Gu et al., 2012). Bruchas and colleagues found that swim stress activated p38 in the brain, particularly GABAergic neurons in the hippocampus, cortex, and NAc (Bruchas et al., 2007). Moreover, 14 days of variate stress, but not a single acute stressor, lead to increased activation of p38 in the amygdala, BNST, and hippocampus. This same stress

procedure also resulted in the p38 regulation of the downstream target GSB3β. Stress and inhibition of GSK3β both result in exaggerated conditioned fear suggesting that the p38 to GSK3β pathway may be involved in the regulation of conditioned fear (Hare 2015). However, to date, no study has examined the role of p38 in conditioned fear. The goal of this study was to examine whether p38 is involved in the acquisition and expression of conditioned fear.

There are four known isoforms of p38 MAPKs, p38α and p38β, p38γ and p38δ. p38α MAPK is the most widely studied; as it is expressed in most cell types and was a target of drugs such as SB203580. SB203580 is a selective inhibitor of p38α and p38β (Cuenda et al., 2007). In addition, research suggests that the isoform, p38α, is required for the downstream signaling action of serotonergic cells for behavioral responses to stress. This underlines p38α ability to selectively target downstream substrates, despite being widespread (Bruchas 2011). As p38α was recently implicated in a pathway activating GSK3β and both of their potentials for implications to stress response, we chose SB203580 to target p38α. Based on the observations that stress regulates p38α and GSK3β and exaggerates conditioned fear and that the genetic inhibition of GSKβ also regulated conditioned fear, we predicted that inhibition of p38 with SB203580 would result in exaggerated conditioned fear.

EXPERIMENT 1

METHOD

Our goal for this experiment was to understand if SB203580 would have an effect on conditioned fear to the tone.

Subjects

Forty-four ale 8-9 week old C57BL6/J mice were obtained from Jackson Laboratory and housed in standard acrylic cages in an AAALAC accredited colony facility. Mice were maintained on a 12-hour light cycle with food and water available ad lib.

Apparatus

Mice were placed in one of 2 identical conditioning chambers (30.5 cm x 24.1 cm x 21 cm), consisting of acrylic walls metal grid floor with no internal light that was scented with Vick's Vaporub (Video Fear Conditioning System MED-VFC-NIR-M, Med-Associates, St. Albans, Vermont). Each chamber was located within a sound-attenuating cubicle. For fear conditioning mice were given 5 tone (4500Hz, 30 sec, 70dB) and foot shock pairings (shocks 20ms 0.5 mA) at a 3 min inter-trial interval. Freezing was assessed using Video Freeze software calibrated on the recommendations of Anagnostaras (Anagnostaras et al., 2002).

Fear to the tone was assessed in an altered context. The altered context was a novel illuminated chamber with brown textured walls and solid floor, scented with anise extract. Freezing was once again assessed using Video Freeze software calibrated on the recommendations of Anagnostaras (Anagnostaras et al., 2002).

Drugs

The p38 MAPK inhibitor, SB203580 was acquired from Tocris Bioscience (Tocris House, IO Centre Bristol, UK) and stored at -20ºC. To dilute the solid form of SB203580, 1 mL of DMSO was added to the 10 mg of SB203580 and vortexed. The solution was then diluted to the final concentration by adding 19 mL of distilled H20. Thus, the final solution was 5% DMSO and the final concentration of SB203580 was 0.5mg/ml. The vehicle solution was a 5%

DMSO in water. Both vehicle and SB were stored in 1 ml aliquots and stored at -20ºC. Injections of SB were given IP at 1 ml/100g for a dose of 5mg/kg.

ANALYSIS

Data are expressed as means +- standard error of the mean. Behavioral analysis utilized mixed model ANOVAs followed by LSD protected t-tests. Results were analyzed by SPSS software version 22 (IBM; Armonk, NY). $p<0.05$ was considered statistically significant.

Procedure

In order to examine whether blocking p38 MAPK would exaggerated conditioned fear, SB203580 was administered prior to auditory fear conditioning. Mice were given 3 intraperitoneal (IP) injections at 6-hour intervals beginning 12.5 hours prior to tone and foot shock fear conditioning. Thorton et al. showed that 3 injections were necessary to inhibit GSK3β. Thirty minutes after the third and last injection, mice were given tone and foot shock fear conditioning. Mice were placed in conditioning chamber and after 1 min given the first of 5 tone and foot shock pairings. A day later mice were tested for fear to the tone in the absence of injections. For testing, mice were placed in the altered context and after 2 min presented with a continuous 3 min tone. % Freezing to the tone was accessed for each 1 min interval of the tone. Freezing to the tone during fear conditioning was assessed by determining the % of the time the mice spent freezing during the tone.

RESULTS

Freezing in the presence of the tone increased over fear conditioning trials ($F(4,168) = 134.542$, $p<0.000$). There were no difference in freezing between groups ($F<1$), and no interaction between training trial and group ($F(4,168)=1.190$, $p = 0.317$). There were no

differences in baseline freezing between the drug groups ($M_{SB} = 0$, $M_{VEH} = 0$). When tested for freezing to the tone 24 hours with SB203580 after fear conditioning, mice injected with SB froze more than those injected with Vehicle. There was an effect due to the compound SB203580, ($F(1,42)=106.903$, $p = 0.019$). Mice froze most during the first minute of the tone with freezing decreasing over the course of the 3-minute tone ($F(2,84)=38.410$, $p < 0.000$). The change in freezing over the test session did not differ between the groups ($F(2,84)=0.195$, $p=0.823$)

EXPERIMENT 2

METHOD

Our goal for this experiment was to examine if SB203580 affects conditioned fear to context.

Subjects

Thirty-one male 8-9 week old C57BL6/J mice were obtained from Jackson Laboratory and housed in standard acrylic cages in an AAALAC accredited colony facility. Mice were maintained on a 12-hour light cycle with food and water available ad lib.

Apparatus

The apparatus was identical to that used in Experiment 1.

Drugs

The drug was identical to that used in Experiment 1.

Procedure

In order study the effect of the p38 MAPK inhibition on contextual fear conditioning, the p38 MAPK inhibitor SB203580 was administered prior to tone and footshock fear conditioning and mice were tested for freezing in the conditioning context one day later. Specifically, mice

were given 3 intraperitoneal (IP) injections at 6-hour intervals beginning 12.5 hours prior to tone and footshock fear conditioning. Thirty minutes after the third and last injection, mice were given tone and footshock fear conditioning as described in Experiment 1. Mice were placed in a conditioning chamber and after 1 min given the first of 5 tone and footshock pairings. A day later mice were tested for fear to the conditioning context in the absence of the tone. % Freezing was measured over 6 continuous 1 minute intervals.

RESULTS

Similar to Experiment 1, mice increased freezing over Tone + Shock trials ($F(4,116)=62.460$, $p<0.000$). There was no difference in tone freezing between groups ($F<1$), and no interaction between training trial and group ($F(4,116)=0.365$, $p<0.833$). There were significant differences of freezing within groups over the 6 minute intervals ($F(5, 145)=2.851$, $p=0.017$) in which it appeared that freezing initially increased then decreased over the 6, 1 minute intervals. However, there was no difference in freezing between mice injected with SB and VEH ($F<1$), and no interaction between minute and drug group ($F(5,145)=.464$, $p=0.803$).

EXPERIMENT 3

METHOD

Our goal for this experiment was to examine how SB203580 affects fear conditioning to the tone, testing to see if it affects acquisition or rather expression of fear to the tone.

Subjects

Forty-six male 8-9 week old C57BL6/J mice were obtained from Jackson Laboratory and housed in standard acrylic cages in an AAALAC accredited colony facility. Mice were maintained on a 12-hour light cycle with food and water available ad lib.

Apparatus

The apparatus was identical to that used in Experiment 1.

Drugs

The drug was identical to that used in Experiment 1.

Procedure

Mice were trained and tested for tone for fear conditioning as described in Experiment 1 with the following exceptions. Mice in the "Pre-Test" group were given injections of SB203580 or vehicle prior to the test for tone freezing in the altered context. For this group, mice were given 3 injections of SB203580 at 6-hour intervals beginning 36 hour after fear conditioning and 12.5 hours before the for tone freezing. The last injection occurred 30 min before the test for tone freezing. Mice in the "Pre-Train" group were given three injections of SB203580 or vehicle at 6-hour intervals with the last injection occurring 30 min before tone and footshock training. Mice in the "Pre-Train" group were then tested for tone freezing in the altered context 48 hours later. Thus, in this experiment, all mice were given fear conditioning and tested for freezing to the tone 48 hours later. The additional 24 hour delay in testing allowed us to assess the effect of SB203580 on the expression of freezing to the tone apart from any effects SB203580 might have on consolidation of fear that might occur in the first 24 hours after tone and footshock training. For purposes of symmetry, testing also occurred 48 hours after training in mice in the Pre-Training group.

RESULTS

Similar to the results of Experiment 1, there were no differences in baseline freezing between drug groups (M_{SB}=0, M_{VEH}=0; see Figure 3), mice increased freezing over Tone + Shock trials as there was a minute difference ($F(4,176)=94.378$, $p<0.000$). There was no difference in tone freezing between treatment groups ($F<1$), and no interaction between training trial and group (Figure3A, $F(4,176)=0.662$, $p=0.629$). There was no difference due to the group, Pre-Train or Pre-Test, (Group difference, $F(1,42)=1.837$, $p=0.183$). There was no difference due to drug treatment ($F<1$). The Pre-Train mice injected with SB203580 froze more than those injected with Vehicle (see figure 3C), but the effect of the drug was not significant (Figure3C, $F(1,22)=52.631$, $p=0.276$).). There was a difference between the 3-minute intervals of the tone, such that Pre-Train mice froze most during the first minute of the tone with freezing decreasing over the course of the 3-minute tone (Figure3B, $F(2,44)=6.355$, $p=0.004$). The change in freezing over the test session did not differ between the Pre-Train treatment groups (See Figure 3C, $F(2,44)=1.653$, $p=0.221$). Pre-Test mice injected with SB froze less than those injected with Vehicle (see figure 3D), but the effect of the drug was not significant (Figure3D, $F(1,20)=28.737$, $p=0.489$). There was a difference between the 3-minute intervals of the tone, such that Pre-Test mice froze most during the first minute of the tone with freezing decreasing over the course of the 3-minute tone (Figure3B, $F(2,40)=20.523$, $p < 0.000$). The change in freezing over the test session did not differ between the Pre-Test treatment groups (Figure3D, $F(2,40)=1.373$, $p=0.265$).

DISCUSSION

The goal of this study was to examine if p38 MAPK inhibition would alter conditioned fear. We found that animals given systemic intraperitoneal (IP) injections of the p38 MAPK inhibiting compound, SB203580, showed greater freezing to the tone. The effect seen in Experiment 1 suggests that the inhibition of p38 MAPK contributes to auditory conditioned fear. SB203580 did not affect freezing to the context. SB203580 did not affect freezing to the tone when tested 48 hours after training, when the injections took place prior to training or testing. It is not known whether the intraperitoneal injections affected p38 MAPK precisely or if peripheral effects mediated the results. Nor is it known whether p38 MAPK was targeted in the brain. It is possible that p38 MAPK was working to affect exaggerated conditioned fear in different regions of the brain or body. As discussed earlier, p38 α is widespread in various cell types. P38α is also known to be involved in the inflammatory response. Further research, such as local injections to specific parts of the brain are necessary to better understand the role of p38 MAPK in conditioned fear.

Also important to note was that the effect of elevated conditioned fear in Experiment 1 was not replicated in Experiment 3. It may be that the timing of the testing in relation to training or lacking sample size contributed to the discrepancy. The exaggerated fear due to pre-training injections of SB seemed to be transient as there was only a significant difference to to the drug when tested 24 hours after training versus 48 hours. Regardless, pre-train SB animals in Experiment 3 did freeze more, and along with the results from Experiment 1; this suggests that the inhibition of p38 MAPK may only affect acquisition of fear memory. Further studies in which the replications of Experiment 3 are needed to raise the power of the experiment. Thus, the acquisition of conditioned fear memory to the tone seems to be primarily affected.

In regards to p38 MAPK and memory, the pro-inflammatory cytokine, interleukin-1β (IL-1β) influences memory consolidation and is mediated by p38. Such a response and hindrance to memory consolidation was limited when SB203580 was used. Physiological levels of IL-1β are considered important for memory formation. However, excessive amounts via stress or pharmacological inductions are considered detrimental (Gonzalez et al., 2013). It is well known that the peripheral nervous system can set off a chain of immunological responses that impact the brain to cause anxiogenic or depressive behaviors (Dantzer et al., 2008). The pro-inflammatory cytokines in the body have been shown to induce not only feelings of sickness but also those underlying major depressive disorders (Dantzer et al., 2008). Previous studies have implicated IL-1β and its effects on the hippocampus for memory formation. It would be useful to target p38 MAPK inhibition in the hippocampus, as well as other memory dependent brain regions and see how that interferes with normal fear conditioning. It may be that our results are reflecting the coordination of p38 MAPK with the immune response, causing a pro-inflammatory effect with cytokine accumulation contributing to elevating conditioned fear. It would be valuable to track the amount of p38 activation and cytokine amounts in the brain before and after fear conditioning. To further study the p38 MAPK pathway on conditioned fear, it may be necessary to potentially induce or inhibit the immune response in conjunction with a p38 inhibitor and fear conditioning, to elucidate how these systems may influence each other.

Research points to differential roles for p38 MAPK in the stress response. Previous studies have shown that acute stress, such as acute restraint, does not activate the p38 MAPK pathways (Shen et al. 2004). Some studies suggest that the inhibition of p38 MAPK attenuates behavioral effects of stress (Réus et al., 2014). Pharmacological inhibition of p38 MAPK decreased the anxiogenic response of immobility seen in forced swim (Bruchas et al., 2007). In

contrast, others show p38 MAPK is naturally reduced during times of stress, and elevated in response to antidepressant drugs (Budziszewska et al., 2010). Our data showed the potential inhibition of p38 caused an anxiety-like behavior, that of freezing. If we interpret the fear conditioning protocol as an acute stressor, then p38 levels should be monitored before and after fear conditioning and in conjunction with a p38 inhibitor. As stress is inevitable linked to the immune response, it would be helpful to also monitor the immune response in stressed and p38 modulated animals. This research indicates the importance of studying p38 MAPK in specific parts of the brain with varying stressors to further understand how it mediates the stress response.

Assuming that SB203580 was working to inhibit phosphorylation of p38 α MAPK, activating a specific cellular pathway and leading to behavioral consequences; it would be useful to further understand p38 MAPK's specific downstream targets. Recently, the Rincon lab identified p38α MAPK as an upstream regulator that inhibits GSK3β by phosphorylation at its C terminus at its antibody Serine 389 (Ser^{389}) in mice, and threonine 390 (Thr^{390}) in humans. Research suggests that p38 MAPKα and GSK3β may be acting in a combined protective effect for cells in response to stress. Interestingly, phosphorylation of GSK3β at Ser^{389} is independent of phosphorylation of its more widely studied Serine9 (Ser9) site. Also important is the fact that Rincon has shown that Ser9 phosphorylation occurs in the cytoplasm whereas Ser^{389} phosphorylation occurs in the nucleus of the neuron and is largely in response to DNA damage (Thornton et al., 2008). Mice lacking the ability to phosphorylate GSK3-at Serine 389 show exaggerated conditioned fear suggesting that phosphorylation of this kinase is important for normal fear learning. The Falls lab has shown that psychological stress increases p38 MAPK and GSK3β Ser^{389} phosphorylation in stress-sensitive areas of the brain, such as the amygdala and hippocampus suggesting that GSK3β Ser^{389} may also play a role in stress. Nuclear p38 activation

increases in times of DNA damage, suggesting p38 MAPK and GSK3β play a role in mediating stress for cell survival (Wood et al., 2009). If the inhibition of p38 lead to increased fear seen with increased immobility, then the p38 pathway and modulation of GSK3β Ser389 may be allowing for exaggerated conditioned fear. It would be necessary to confirm, with the use of Western Blots, what proteins and cells are being activated and where in the brain, when a p38 MAPK inhibitor is used during fear conditioning. Lastly, the immune response would be helpful to monitor as this pathway may be linked. This would be helpful in illuminating the potential protein pathways that contributed to the acquisition and expression of conditioned fear.

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Figure 1.

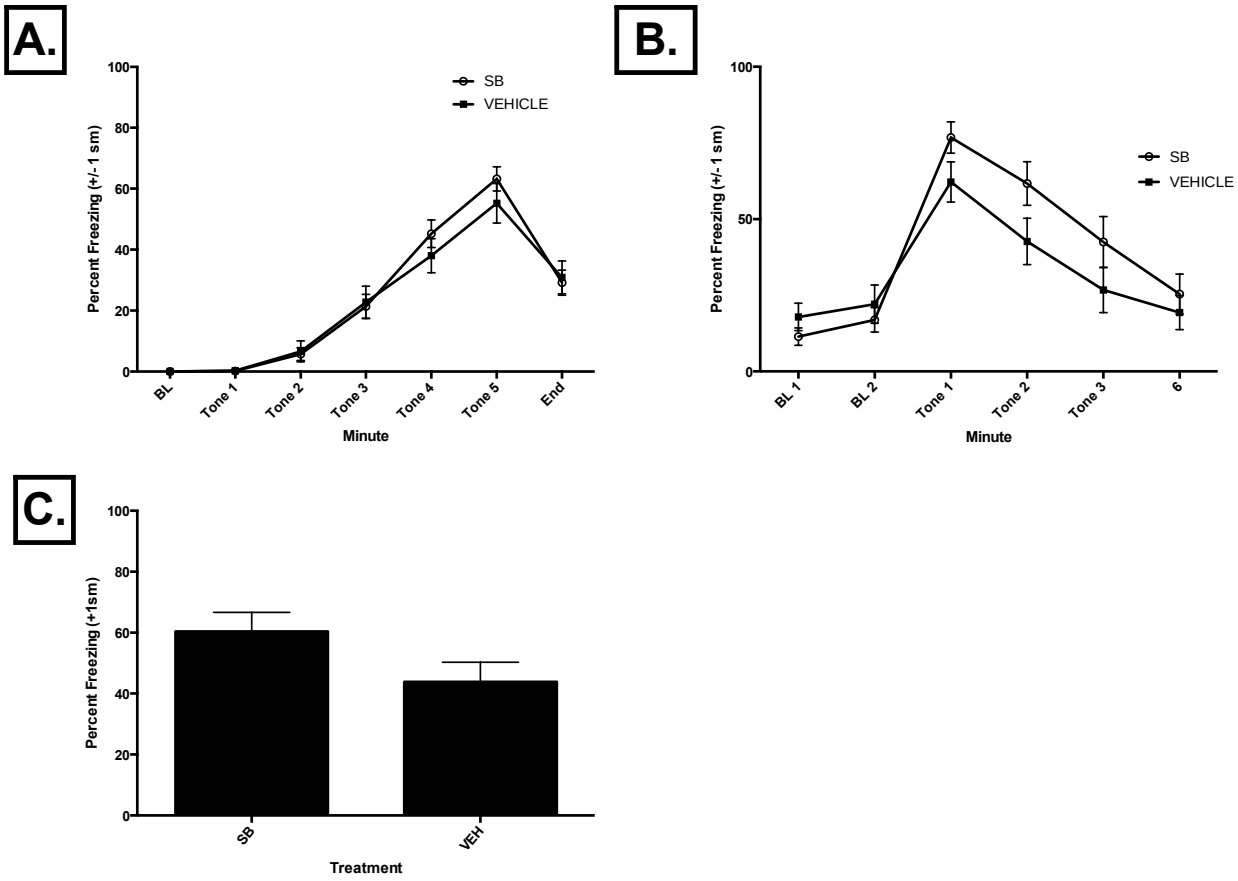

Figure 1. Pre-training intraperitoneal (IP) injection of SB203580 increased auditory fear conditioning tested 24 hours after tone + shock.
A. Freezing to the tone during tone + shock training
B. Mice injected with SB203580 prior to the tone + shock fear conditioning showed elevated freezing over entire three minutes of the tone.
C. Mice injected with SB203580 prior to the tone + shock showed elevated freezing over the course of the 3 minute tone. There was no difference in freezing prior to the onset of the tone.

Figure 2.

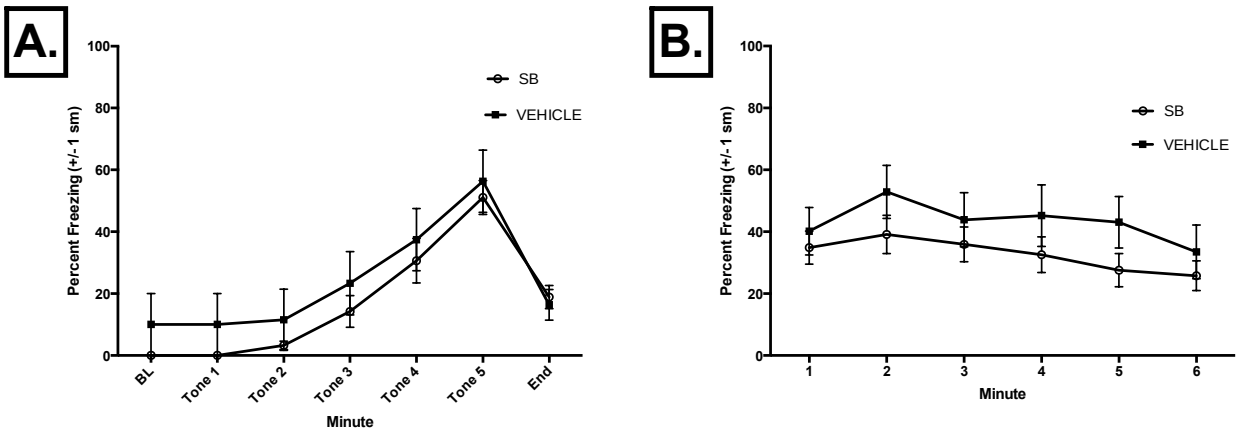

Figure 2. Pre-Training intraperitoneal (IP) injection of SB203580 did not alter contextual fear conditioning.
A. Freezing to the tone during tone + shock training
B. There was no difference in freezing to the training context between mice injected with either SB203580 or vehicle over the course of 6 minutes.

Figure 3.

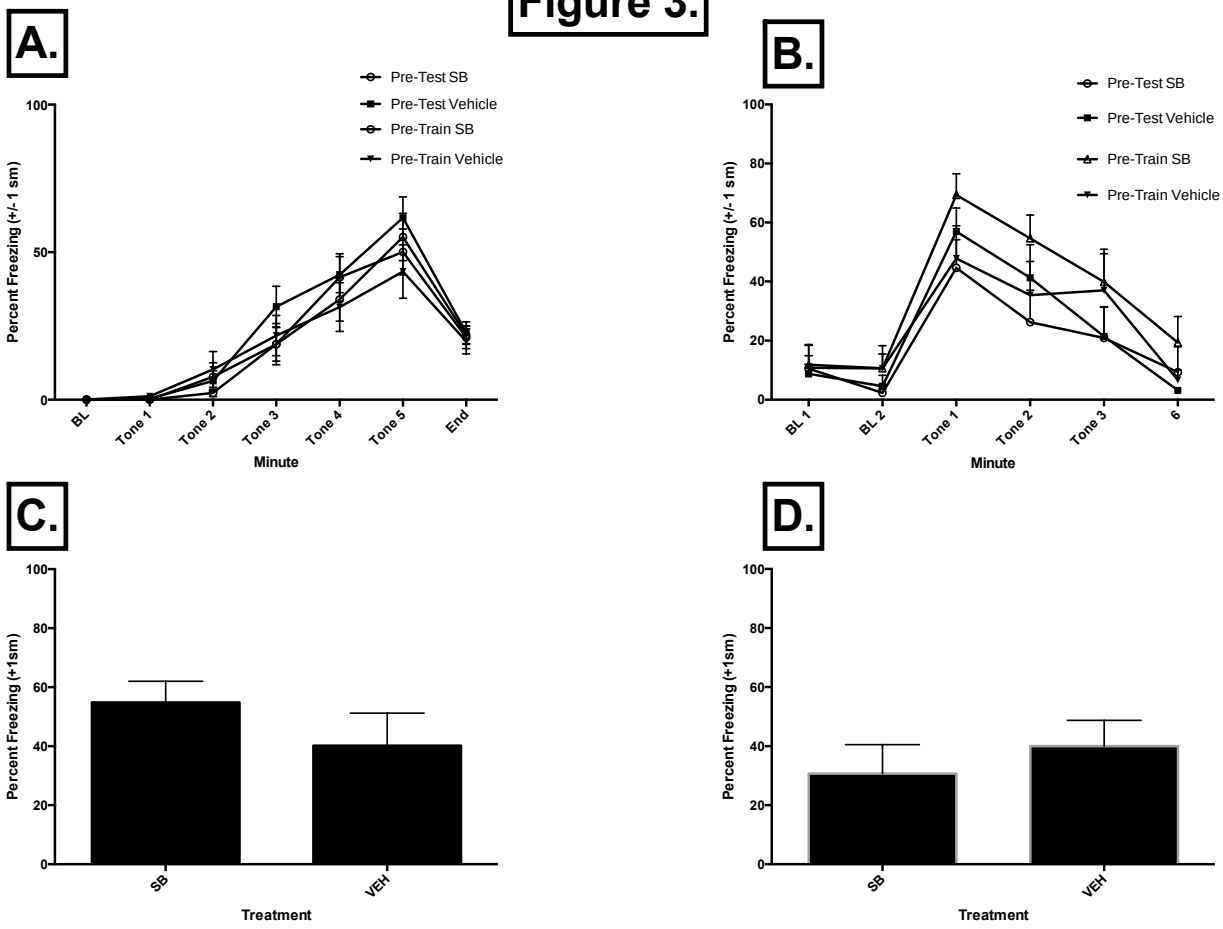

Figure 3. Pre-training and Pre-testing intraperitoneal (IP) injection of SB203580 did not increase auditory fear conditioning when tested 48 hours after fear conditioning.
A. Freezing to the tone during tone + shock training
B. Freezing to the tone was not significantly different for all four groups when tested 48 hours after training.
C. Mice injected with SB203580 prior to the tone + shock fear conditioning showed elevated freezing over entire three minutes of the tone.
D. Mice injected with vehicle prior to testing showed elevated freezing over entire three minutes of the tone.