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Stronger Learning Recruits Additional Cell-Signaling Cascades: c-Jun-N-Terminal Kinase 1 (JNK1) is Necessary for Expression of Stronger Contextual Fear Conditioning

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Abstract

Increased training often results in stronger memories but the neural changes responsible for these stronger memories are poorly understood. It is proposed here that higher levels of training that result in stronger memories recruit additional cell signaling cascades. This study specifically examined if c-Jun n-terminal kinase 1 (JNK1) is involved in the formation of stronger fear conditioning memories. Wildtype (WT), JNK1 heterozygous (Het), and JNK1 knockout (KO) mice were fear conditioned with 1 trial, 2 trials, or 4 trials. All mice learned both contextual (hippocampus-dependent) and cued (hippocampus-independent) fear conditioning but for contextual fear conditioning only, the JNK1 KO mice did not show higher levels of learning with increased trials. That is, WT mice showed a significant linear increase in contextual fear conditioning as training trials increased from 1 to 2 to 4 trials whereas KO mice showed the same level of contextual fear conditioning as WT mice for 1 trial training but did not have increased levels of contextual fear conditioning with additional trials. These data suggest that JNK1 may not be critical for learning but when higher levels of hippocampus-dependent learning occur, JNK1 signaling is recruited and is necessary for stronger hippocampus-dependent memory formation.

1. Introduction

Long-term memory formation involves the recruitment of intracellular signaling cascades that regulate mRNA expression and de novo protein synthesis (Abel, Nguyen, Barad, Deuel, Kandel, and Bourchouladze, 1997; Bourchouladze, Abel, Berman, Gordon, Lapidus, and Kandel, 1998; Bourchouladze, Frenguelli, Blendy, Cioffi, Schutz, and Silva, 1994; Schafe,

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Nadel, Sullivan, Harris, and LeDoux, 1999). It is clear that with increased training, stronger learning can occur (Bourtchouladze et al., 1998; Carew, Walters, and Kandel, 1981). However, it is unclear whether the cellular processes involved in learning change as learning becomes stronger. One possibility is that with increased training, the same cellular substrates are activated but perhaps in greater amount and/or with a different timing. Another possibility is that with increased training, additional cell-signaling cascades are recruited and the activation of these cascades contributes to the formation of stronger memories.

There are multiple ways to strengthen learning that include increased training and pharmacological manipulation. For instance, acute nicotine enhances learning and prior work suggests that hippocampus-dependent learning is particularly sensitive to the effects of acute nicotine (Davis, James, Siegel, and Gould, 2005; Gould and Lommock, 2003; Gould and Wehner, 1999; Gulick and Gould, 2008; Portugal, Wilkinson, Kenney, Sullivan, and Gould, 2012; Portugal, Wilkinson, Turner, Blendy, and Gould, 2012). This enhancement was associated with an increase in c-Jun N-terminal kinase 1 (JNK1) expression and JNK (46 kD) activation, and inhibition of JNK (non-selective) blocked the enhancement of learning by nicotine and yet the same dose did not block learning in nicotine-naïve mice (Kenney, Florian, Portugal, Abel, and Gould, 2010). JNK1 has been associated with apoptosis (Ham, Eilers, Whitfield, Neame, and Shah, 2000) and development of the brain (Gelderblom, Eminel, Herdegen, and Waetzig, 2004) but the results from the studies with nicotine also suggest that activation of JNK1 may modulate learning. JNK has cytoplasmic and nuclear effectors (Barr and Bogoyevitch, 2001; Chang, Jones, Ellisman, Goldstein, and Karin, 2003; Eminel, Roemer, Waetzig, and Herdegen, 2008; Kim, Futai, Jo, Hayashi, Cho, and Sheng, 2007; Thomas, Lin, Nuriya, and Huganir, 2008; Weston and Davis, 2002), making it a versatile signaling molecule. Furthermore, JNK1 interacts with many cytoplasmic proteins that are critical to learning and memory processes. For instance, JNK1 is important for AMPA receptor trafficking to the membrane (Thomas et al., 2008), PSD-95 phosphorylation (Kim et al., 2007), neurite outgrowth (Eminel et al., 2008), and microtubule associated protein activation and polymerization (Chang et al., 2003). Thus, it is possible that stronger learning recruits JNK1 signaling.

The current study tested the hypothesis that JNK1 is necessary when stronger learning occurs. Wildtype (WT), JNK1 heterozygous (Het), and JNK1 knockout (KO) mice were tested in contextual fear conditioning and cued fear conditioning. In addition to varying genotype, the level of training was varied. Each genotype was trained with either 1 trial, 2 trials, or 4 trials. If JNK1 is involved with higher levels of learning, it was predicted that learning would remain asymptotic across number of conditioning trials in the JNK1 KO mice but would increase with increased trials in the wildtype mice.

2. Methods

2.1. Subjects

Male and female JNK1 mutant mice (8-12 weeks of age originally created by Dong and colleagues (1998)) were obtained from Jackson Laboratory (Bar Harbor, ME) and bred at Temple University. All breeding was carried out using heterozygous by heterozygous matings. Mice were weaned at 3-4 weeks of age into group housing conditions (2-4 per

cage) with same sex littermates. Mice were maintained in a temperature and humidity controlled vivarium and allowed *ad libitum* access to food and water.

2.2. Fear Conditioning Procedures

Male and female mice were trained in modular fear conditioning chambers (18 × 19 × 38 cm) contained in sound attenuating cubicles (Med Associates, St Albans, VT). Subjects were placed into the fear conditioning chamber and allowed to explore for 120 seconds. After the 120 second baseline period, a white noise conditioned stimulus (CS) played for 30 seconds and co-terminated with a 2 second shock (0.57 mA) delivered through the grid floor which acted as an unconditioned stimulus (US). Separate groups of mice either received 1 training session that consisted of either 1 trial (WT n=9; Het n=14; KO n=12), 2 trials (WT n=9; Het n=14; KO n=10), or 4 trials (WT n=7; Het n=11; KO n=9). The intertrial interval for multiple trial sessions was 120 seconds. After the last trial, the mice remained in the chamber with the chamber lights on for 30 seconds. Twenty-four hours later, mice were assessed for freezing behavior in the training context for five minutes. One hour after contextual fear conditioning testing, mice were placed into an altered context and assessed for freezing in the novel environment for 3 minutes before the CS was introduced for an additional 3 minutes. The altered context chambers (20.30 × 22.90 × 17.80 cm) were located in a different room and had different visual, tactical, and olfactory cues than the conditioning chambers.

2.3. Western Blotting Procedures

To confirm the absence of JNK1 protein in the JNK1 KO mice, hippocampal homogenates from JNK1 WT and KO mice were subjected to standard SDS-PAGE and western blotting to verify the presence and absence of JNK1 protein levels, respectively. Briefly, whole hippocampi from JNK1 WT and KO mice were sonicated in homogenization buffer A (Sambrook, Fritsch, and Maniatis, 1989) and total protein level was verified using DC Protein Assay (Bio-Rad, Hercules, CA). 10 µg protein was added to each well of a polyacrylamide gel (12%) and samples were subjected to electrophoresis to separate the protein bands. After transferring to a nitrocellulose membrane using a standard wet transfer, immunoblotting was conducted using a mouse monoclonal antibody specific for JNK1 (BD Pharmingen, San Jose, CA) diluted 1:1000 in Tris-buffered saline with Tween (T-TBS) containing 5% non-fat dry milk, and incubated at 4 degrees C overnight. After primary antibody incubation, blots were washed in T-TBS prior to incubation with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse) diluted 1:10000 in T-TBS. Blots were developed with ECL (Pierce, Thermo Scientific, Rockford, IL) and imaged using a Gel Logic 1500 imager (Kodak, Rochester, NY).

2.4. Statistics

Results were analyzed using GraphPad Prism 5 statistical software. Fear conditioning results were analyzed using 3 × 3 factorial ANOVAs with genotype (WT, Het, and KO) and training paradigm (1, 2, and 4 CS-US pairings) as between subject factors. Separate ANOVA analyses were conducted for contextual and cued fear conditioning. Bonferroni corrected planned comparisons were used to determine genotype differences at each level of

fear conditioning training. Since it would be expected that increased strength of training would increase freezing levels in both contextual and cued conditioning, each genotype was also evaluated for linear trend contrast analysis with Bonferroni correction.

3. Results

3.1. Fear Conditioning

JNK1 KO mice expressed similar levels of contextual fear conditioning as WT mice when trained with 1 CS/US pairing but not with 2 and 4 CS/US pairings (Figure 1). A two-way ANOVA of contextual fear conditioning with genotype (WT, Het, and KO) and trial number (one, two, and four) as between-subjects factors revealed significant main effects of genotype [$F(2, 86) = 10.53$, $p < 0.05$] and trial number [$F(2, 86) = 3.979$, $p < 0.05$]; but no interaction [$F(4, 86) = 1.037$, $p > 0.05$]. Bonferroni corrected a priori analyses revealed that KO mice exhibited significantly less contextual fear conditioning than WT when trained with 2 CS/US pairings or 4 CS/US pairings, but not when trained with 1 CS/US pairing: $p < 0.05$. KO mice froze significantly less than Het mice at 2 CS/US pairings only: $p < 0.05$.

There were no overall effects of JNK1 gene dosage on freezing to the auditory cue. A two-way ANOVA of cued freezing (with the same factors as above) revealed no effect of genotype [$F(2, 86) = 0.0780$, $p > 0.05$] but a significant main effect of trial number [$F(2, 86) = 9.467$, $p < 0.05$]. No significant interaction was found [$F(4, 86) = 1.527$, $p > 0.05$]. Bonferroni corrected a priori analyses revealed no significant differences between WT and KO or WT and Het mice. There were no observed effects of genotype, trial number, or interactions during altered context (pre-CS) freezing.

Studies have shown that strength of training increases fear conditioning responses (Bourtchouladze et al., 1998; Maren, 1999; Wiltgen, Sanders, Anagnostaras, Sage, and Fanselow, 2006). WT mice showed significant linear trends, revealing strength of training-dependent increases in both contextual and cued fear conditioning (Slope=13.02 and $r^2=0.2901$, $p < 0.05$; slope=15.96 and $r^2=0.3777$, $p < 0.05$ respectively, Figure 2). Het mice did not have a significant linear trend for contextual conditioning but did show a linear trend for cued fear conditioning (Slope=3.571 and $r^2=0.02597$, $p > 0.05$; slope=9.145 and $r^2=0.1276$, $p < 0.05$, respectively, Figure 2). KO mice did not have a linear trend for either contextual or cued fear conditioning (Slope=2.963 and $r^2=0.01763$, $p > 0.05$; slope=6.25 and $r^2=0.07162$, $p > 0.05$ respectively, Figure 2). The lack of a linear trend for cued fear conditioning in the KO mice was due to a higher level of cued fear conditioning with 1 trial training, which is why the KO mice did not have a deficit in cued fear conditioning at any level of conditioning. Specifically, regardless of the number of conditioning trials, KO mice exhibited similar levels of cued fear conditioning ($60\% \pm 7$, $63\% \pm 4$, and $72\% \pm 5$; 1, 2, or 4 trials respectively) in contrast to WT and Het mice (WT: $45\% \pm 5$, $73\% \pm 6$, $77\% \pm 5$; Het: $56\% \pm 5$, $60\% \pm 6$, $74\% \pm 4$; 1, 2, or 4 trials respectively). For contextual fear conditioning, KO and Het mice exhibited similar levels of freezing within genotype regardless of the strength of training protocol (KO: $41\% \pm 6$, $41\% \pm 5$, $47\% \pm 7$; Het: $56\% \pm 4$, $63\% \pm 6$, $63\% \pm 5$; 1, 2, or 4 trials respectively), whereas WT mice showed a clear increase in contextual fear conditioning as the number of shocks increased ($48\% \pm 6$, $65\% \pm 5$, $74\% \pm 8$; 1, 2, or 4 trials respectively).

3.2. Western Blotting

Hippocampi from JNK1 WT mice had detectable levels of JNK1 protein, whereas KO mice did not (Figure 3). Samples from 3 WT and 3 KO revealed consistent and reproducible effects.

4. Discussion

The present study found that while JNK1 may not be necessary for fear conditioning, JNK1 is necessary for stronger contextual fear conditioning but not for stronger cued fear conditioning. JNK1 KO and WT mice showed similar levels of contextual fear learning after 1 trial. However, JNK1 KO mice showed significantly less contextual fear conditioning than WT mice when training consisted of 2 or 4 trials. This was due to the JNK1 KO mice showing asymptotic levels of contextual fear conditioning across 1, 2 and 4 trial training while WT mice showed a significant linear increase in contextual fear conditioning with increasing numbers of trials. Wildtype and JNK1 KO mice also showed similar levels of cued fear conditioning when trained with 1, 2 or 4 trials. Because contextual fear conditioning is hippocampus-dependent while cued fear conditioning is not (Kim and Fanselow, 1992; Logue, Paylor, and Wehner, 1997; Phillips and LeDoux, 1992), the current results suggest that stronger levels of hippocampus-dependent learning may require activation of JNK1. In addition, these results further demonstrate that the cell signaling molecules involved in hippocampus-dependent learning are not necessarily involved in hippocampus-independent learning as no significant effects were seen on cued fear conditioning; however, it remains untested if an effect on cued fear conditioning would be seen if a weaker CS was used or if cued freezing was assessed without contextual freezing assessed beforehand.

It appears that stronger learning is not mediated by identical cellular processes that support lower levels of learning. This is supported by prior work and the current results. It was previously found that fear conditioning with 1 CS-US pairing recruited two periods of PKA-dependent protein synthesis during consolidation whereas a stronger training paradigm with 3 CS-US pairings recruited only one period of PKA-dependent protein synthesis during consolidation (Bourtchouladze et al., 1998). While those results do not demonstrate that stronger learning recruits different cell signaling cascades, they do show that the underlying cellular and molecular substrates of learning change with higher levels of learning. The present results suggest that different signaling pathways, such as the JNK1 pathway, may be recruited to form stronger memories but that these signaling pathways may not be necessary in situations where a more modest level of learning occurs.

There has been some discrepancy regarding the effect of JNK inhibition on learning, but those differences may be due to differences in the level of training used in the conflicting studies. In a prior study, we found that that infusion of the pan JNK (i.e., JNK1, JNK2, JNK3) inhibitor SP600125 (20 μ M) directly into the dorsal hippocampus during consolidation (i.e., after training) produced a deficit in contextual fear conditioning (Kenney et al., 2010). Another study, however, reported that infusion JNK SP600125 (30 μ M) into the dorsal hippocampus during consolidation did not disrupt contextual fear conditioning and instead increased freezing (Sherrin, Blank, Hippel, Rayner, Davis, and Todorovic,

2010). Differences in training procedures may explain the discrepant results as the Sherrin study used one trial contextual fear conditioning and the Kenney study used 2 trial contextual fear conditioning. Given the current data that suggest that the involvement of JNK1 in contextual fear conditioning changes with the level of training, the different results from the two studies might be because Sherrin and colleagues used a training paradigm that does not require JNK1 but Kenney and colleagues' study used a training paradigm that did. If JNK1 is recruited with stronger learning, this may also explain why JNK1 is involved in the nicotine enhancement of learning (Gould and Higgins, 2003; Gould and Wehner, 1999). Nicotine produces stronger hippocampus-dependent learning (Gould and Leach, 2014; Kenney and Gould, 2008), similar to what is seen with more learning trials, and thus JNK1 may be activated by nicotine during learning for the formation of stronger memories.

An important direction for future studies is to identify the mechanisms through which JNK1 activation could contribute to stronger memory formation. JNK1 activates many transcription factors including JUN family, ATF-2, and Elk-1 (Bogoyevitch and Kobe, 2006; Gupta, Barrett, Whitmarsh, Cavanagh, Sluss, Derijard, and Davis, 1996) that are associated with learning and plasticity. Specifically, hippocampal long-term depression (LTD) was disrupted in JNK1 KO mice and this disruption was associated with decreased activation of c-Jun and ATF-2 (Li, Li, Yu, Chen, Sabapathy, and Ruan, 2007). Other studies, while not examining JNK1, have shown a critical role of Elk-1 and JunB in learning. For example, contextual fear conditioning was associated with an increase in Elk-1 activation in the CA3 region of the hippocampus (Sananbenesi, Fischer, Schrick, Spiess, and Radulovic, 2002). This increase in Elk-1 was associated with an increase in ERK1/2 activation, which was proposed to be a critical component of contextual fear memory consolidation. In addition, increased JunB was seen in the dorsal hippocampus after contextual fear conditioning (Strekalova, Zorner, Zacher, Sadvoska, Herdegen, and Gass, 2003). Thus, one possible mechanism through which JNK1 could strengthen memory is through increasing activation of transcription factors such as JUN family, ATF-2, and Elk-1.

In addition to activating transcription factors associated with learning and plasticity, JNK1 interacts with many cytoplasmic proteins that are critical to learning and memory processes. For instance, JNK1 is important for AMPA receptor trafficking to the membrane (Thomas et al., 2008), PSD-95 phosphorylation (Kim et al., 2007), neurite outgrowth (Eminel et al., 2008), and microtubule associated protein activation and polymerization (Chang et al., 2003); all of which can contribute to memory formation. AMPA receptor trafficking has been proposed as a fundamental mechanism of learning and plasticity (for review see Malinow and Malenka, 2002; Stern and Alberini, 2013). PSD-95 is a scaffolding protein that strengthens synapses in part through increasing surface levels of AMPA receptors and JNK1 signaling was associated with increased synaptic accumulation of PSD-95 (Kim et al., 2007). Neurite outgrowth has been proposed to be critically involved in synaptogenesis (for review see Pfenninger, de la Houssaye, Helmke, and Quiroga, 1991) and changes in microtubule associated proteins in the hippocampus have been proposed to be an important part learning-related plasticity (Fanara P1, Husted KH, Selle K, Wong PY, Banerjee J, Brandt R, Hellerstein, 2010). Thus, there are several pathways through which JNK1 could contribute to memory formation. If it is a goal of science to not only understand the neurobiology of learning but to also facilitate the development of treatments for cognitive

disorders, then it will be important to also identify the neural substrates that enable the formation of stronger memories.

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Highlights

- Stronger learning recruits additional cell signaling cascades.
- JNK1 is differentially involved in weak versus strong learning.
- WT, but not JNK1 KO, mice show increased contextual memory with stronger training.
- JNK1 WT and Het mice show increased cued memory in response to stronger training.

Fig 1.

The effect of training strength on contextual and delay-cued fear conditioning in JNK1 WT, Het, and KO mice. JNK1 KO mice exhibit selective deficits at moderate (2 CS-US Pairings) and strong (4 CS-US Pairings) training paradigms, but are normal under weak (1 CS-US Pairing) training conditions. * indicates significantly different than WT control ($p < 0.05$).

Fig 2.

Linear trend analyses across the different JNK1 genotypes. WT mice exhibited a significant linear trend of training strength in contextual and cued fear conditioning. Het mice exhibited a significant linear trend of training strength in cued fear conditioning, but not in contextual fear conditioning. KO mice exhibited no linear trend of training strength in either fear conditioning paradigm. → and * indicates significant linear trends ($p < 0.05$).

Fig 3.
Genotype confirmation by western blot analysis. Blots were probed with JNK1 specific antibody. Wells contain lysate from JNK1 WT and KO (3 per genotype).