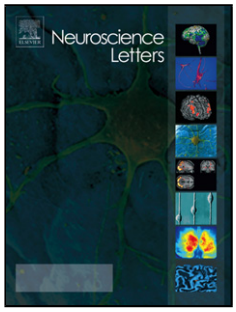

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PII: S0304-3940(16)30376-7
DOI: http://dx.doi.org/doi:10.1016/j.neulet.2016.05.048
Reference: NSL 32067

To appear in: *Neuroscience Letters*

Received date: 19-2-2016
Revised date: 23-5-2016
Accepted date: 24-5-2016

Please cite this article as: Prescott T.Leach, Justin W.Kenney, Thomas J.Gould, c-Jun-N-Terminal Kinase 1 (JNK1) is Necessary for Nicotine-Induced Enhancement of Contextual Fear Conditioning, Neuroscience Letters http://dx.doi.org/10.1016/j.neulet.2016.05.048

c-Jun-N-Terminal Kinase 1 (JNK1) is Necessary for Nicotine-Induced Enhancement of Contextual Fear Conditioning

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Highlights

- JNK1 is critical for nicotine-enhanced contextual fear conditioning.
- JNK1 WT mice show increased contextual memory in response to nicotine treatment.
- JNK1 KO mice do not exhibit nicotine-enhanced contextual memory.

Abstract

Acute nicotine enhances hippocampus-dependent learning. Identifying how acute nicotine improves learning will aid in understanding how nicotine facilitates the development of maladaptive memories that contribute to drug-seeking behaviors, help development of medications to treat disorders associated with cognitive decline, and advance understanding of the neurobiology of learning and memory. The effects of nicotine on learning may involve recruitment of signaling through the c-Jun n-terminal kinase family (JNK 1-3). Learning in the presence of acute nicotine increases the transcription of mitogen-activated protein kinase 8 (Mapk8, also known as JNK1), likely through a CREB-dependent mechanism. The functional significance of JNK1 in the effects of acute nicotine on learning, however, is unknown. The current studies undertook a backward genetic approach to determine the functional contribution JNK1 protein makes to nicotine-enhanced contextual fear conditioning. JNK1 wildtype (WT) and knockout (KO) mice were administered acute nicotine prior to contextual and cued fear conditioning. 24 hours later, mice were evaluated for hippocampus-dependent (contextual fear conditioning) and hippocampus-independent (cued fear conditioning) memory. Nicotine selectively enhanced contextual conditioning in WT mice, but not in KO mice. Nicotine had no effect on hippocampus-independent learning in either genotype. JNK1 KO and WT mice given saline showed similar levels of learning. These data suggest that JNK1 may be recruited by nicotine and is functionally necessary for the acute effects of nicotine on learning and memory.

Keywords: acetylcholine, mitogen-activated protein kinase, hippocampus, addiction, Alzheimer's disease, cognition, genetics

1. Introduction

Nicotine has lower reinforcing properties than many other drugs of abuse, yet it has a very high addictive liability [14]. Recent theories suggest that aside from reinforcing effects, nicotine possesses other unique qualities that contribute to its high rate of use. Some examples of these unique properties include effects of nicotine on attention, learning, and memory [10]. Specifically, acute nicotine administration enhances attention [32], reduces impulsivity [18], and enhances hippocampus-dependent learning [11].

To understand how the effects of acute nicotine on cell signaling contribute to enhanced learning, initial studies have evaluated some of the most well-studied learning and memory-related molecules. Extracellular regulated kinase 1 and 2 (ERK1/2), the prototypical mitogen activated protein kinases (MAPKs), play an important role in contextual learning [2]; and are also important for nicotine's effects on contextual fear conditioning as decreasing ERK1/2 activation prevented nicotine enhancement of contextual learning [27]. Similarly, protein kinase A (PKA) plays an integral role in long-term memory formation [1] and decreasing PKA activity blocked nicotine enhancement of hippocampus-dependent learning [12]. Furthermore, acute nicotine shifts the timing of learning-related PKA and ERK1/2 activation in the dorsal hippocampus [12]. Thus, it is likely that acute nicotine affects the delicate balance of intracellular signaling, leading to stronger learning.

Recently, c-jun n-terminal kinases (JNKs), another branch of the MAPK family (MAPK8-10), have been implicated in learning and memory processes [20, 28]. Specifically, our work suggests that more robust associative conditioning induced by additional training trials may recruit JNK1 activity and contribute to stronger learning [20]. In addition, we previously showed that JNK is involved in the effects of nicotine on acquisition of contextual fear conditioning, but the use of a pan JNK inhibitor (JNK1-3) did not allow the specific JNK subtype to be verified, although it was presumably JNK1-specific [15]. The work of Kenney and colleagues evaluated nicotine-induced alterations in *Jnk1* gene expression [15] and transcription factor activity, including CREB, at the *Jnk1* promotor [16], but these studies did not determine the functional role JNK1 protein plays in nicotine-enhanced hippocampus-dependent learning. In order to directly test for the

involvement of JNK1 protein in nicotine-enhanced learning, JKN1 knockout (KO) and wildtype (WT) mice were evaluated for contextual and cued fear conditioning after acute nicotine treatment.

2. Methods

2.1. Subjects

Heterozygous male and female JNK1 breeder mice [8] were obtained from the Jackson Laboratory (Bar Harbor, ME; Stock # 004319) and bred at Temple University. Preliminary analysis of JNK1 WT mice revealed elevated freezing in a standard fear conditioning paradigm [20]. In initial studies, JNK1 KO and WT mice showed inordinately high levels of freezing. In order to reduce the chances of a ceiling effect, mice were backcrossed to C57BL/6 (Jackson Laboratories) for three additional generations (N3), which brought WT freezing in-line with inbred C57BL/6J freezing performance (data not shown). Subsequent to this event, the Jackson Laboratory conducted SNP analysis and found that two of five markers that determine C57BL/6J from C57BL/6N were segregating, suggesting the original founder mice were likely on a mixed C57BL/6J x C57BL/6N genetic background (http://jaxmice.jax.org/strain/004319.html). Interestingly, C57BL/6J and C57BL/6N significantly differ in contextual fear conditioning performance [4], which lends support to our strategy for reducing freezing levels by backcrossing our mice for 3 additional generations to C57BL/6J. All breeding for behavioral analysis was carried out using heterozygous by heterozygous matings, which were conducted after the completion of backcrossing. All mice were weaned at ~3 weeks of age into same sex (littermate) cages and were maintained in a temperature and humidity controlled vivarium with *ad libitum* access to standard lab chow and water. 67 WTs, including 30 vehicle treated, 11 treated with 0.09 mg/kg nicotine, 11 treated with 0.18 mg/kg nicotine, and 15 treated with 0.36 mg/kg nicotine, and 37 KOs, including 14 vehicle treated, 8 treated with 0.09 mg/kg nicotine, 8 treated with 0.18 mg/kg nicotine, and 7 treated with 0.36 mg/kg, were used in the present experiments.

2.2. Drugs and administration:

(-) Nicotine hydrogen tartrate (Sigma, reported as freebase weight) was dissolved in physiological saline. All doses were administered intraperitoneally (IP) at a dose volume of 10 mL/kg. Acute nicotine (0,

0.09, 0.18, or 0.36 mg/kg) was administered to mice 5 minutes prior to the initiation of training and both testing sessions (context and cued). Nicotine doses were based on a dose found to produce plasma nicotine levels similar to those of human smokers [6] and subsequent dose response curve analysis.

2.3. Contextual and cued fear conditioning

Mice were trained via LabView software in fear conditioning chambers (18 × 19 ×38 cm) in sound attenuating cubicles (Med Associates, St Albans, VT). A 30 second white noise was used as a conditioned stimulus (CS) and a 2 second scrambled foot shock (0.57 mA) delivered through the grid floor acted as an unconditioned stimulus (US). Subjects were placed into the conditioning chambers and allowed to explore for 120 seconds before receiving 2 CS-US pairings, each separated by an inter-trial-interval of 120 seconds. The mice remained in the chambers with the chamber lights on for 30 seconds after the second CS-US pairing. Twenty-four hours later, mice were returned to the conditioning chambers and were assessed for freezing behavior (contextual freezing). One hour later, mice were assessed for freezing in an altered context for 3 minutes prior to CS presentation (pre cue freezing) and freezing was assessed for an additional 3 minutes while the CS was presented (cued freezing). 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.

2.4. Statistical analysis

Results were analyzed using GraphPad Prism 5 statistical software. Fear conditioning results were analyzed using 2 X 4 factorial ANOVAs with genotype (WT and KO) and nicotine dose (0, 0.09, 0.18, and 0.36 mg/kg, IP) as between subject factors. Separate ANOVA analyses were conducted for contextual and cued fear conditioning. Bonferroni-corrected comparisons were used to determine nicotine's effects within each genotype and if genotype differences occurred at each nicotine dose.

3. Results

Nicotine treatment significantly affected contextual fear conditioning in WT mice, but not JNK1 KOs **(Figure 1)**. A two-way ANOVA of contextual fear conditioning with genotype (WT and KO) and nicotine

dose (0, 0.09, 0.18, and 0.36 mg/kg, IP) as between-subjects factors revealed a significant main effect of genotype (WT freezing > KO freezing) [$F(1,96)=18.42$, $p<0.0001$]; no effect of nicotine dose [$F(3,96)=1.809$, $p=0.1506$]; and a significant interaction [$F(3,96)=7.711$, $p=0.0001$]. Bonferroni corrected analyses revealed that WT mice exhibited significantly more freezing than KO mice at 0.18 and 0.36 mg/kg ($p<0.05$) and that WT mice exhibited significantly more freezing at 0.36 than vehicle treated mice.

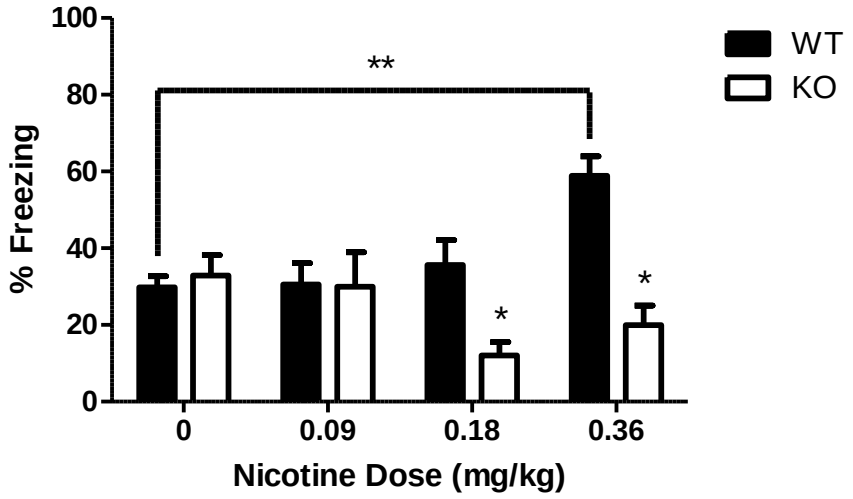

Fig 1. The effect of nicotine on contextual fear conditioning in JNK1 WT and KO mice. Nicotine enhanced performance of JNK1 WT at 0.36 mg/kg, but had no effect at any dose in KO mice. JNK1 KO mice exhibit deficits at 0.18 and 0.36 mg/kg compared to WT mice. * indicates significantly different doses between WT and KO ($p<0.05$) and ** indicates significantly different from vehicle-treated group ($p<0.05$) within genotype.

Nicotine had no effect on cued fear conditioning (**Figure 2**) as assessed by freezing to the auditory cue. A two-way ANOVA of cued freezing (with the same factors as above) revealed a main-effect of genotype (WT freezing > KO freezing) [$F(1,96)=5.131$, $p=0.0258$]; no effect of nicotine [$F(3,96)=1.704$, $p=0.1714$]; and no interaction [$F(3,96)=0.4209$ $p=0.7384$]. There is the potential for ceiling effects in cued fear conditioning, in that all groups froze near 65%. However, recent studies have shown that ceiling effects do not contribute to the absence of nicotine's effects on the hippocampus-independent fear memory. In these

prior studies, we have seen cued fear conditioning as high as >85% and we determined that nicotine did not affect cued conditioning when more modest training occurred, which suggests there is not a ceiling effect and indicates that enhancement could be detected [9]. Therefore, nicotine could still have increased cued fear conditioning in the present study, but this was not seen. Nicotine's selective effects on contextual fear conditioning is in line with previous work [3, 7, 9, 11, 13, 15, 19, 25, 26], but see [22].

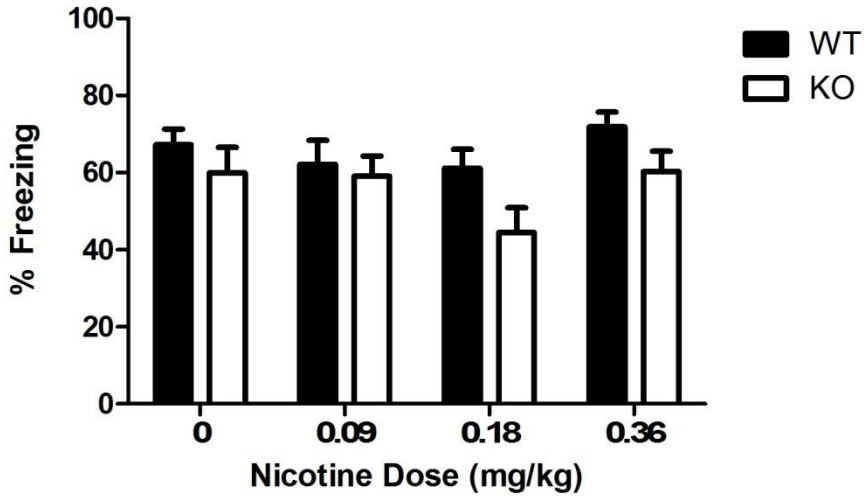

Fig 2. The effect of nicotine on cued fear conditioning in JNK1 WT and KO mice. Nicotine did not affect performance of JNK1 WT or KO mice.

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4. Discussion

The present study revealed a specific role for JNK1 protein in nicotine-enhanced hippocampus-dependent memory. While nicotine produced the expected enhancement of contextual fear conditioning in WT mice, no such effect was observed in JNK1 KO mice. These findings are in line with prior work [20] showing that JNK1 may be recruited during periods of stronger learning activity. The current and prior work highlights that there may be multiple ways to strengthen memory traces: by training with more trials [20] or through the administration of nicotine, and that both may recruit JNK1 activity. While the higher doses of nicotine appeared to negatively affect learning in the KO mice (i.e., decrease freezing), this was unlikely due to off-target activity. In support of this, there were no effects on cued fear conditioning, which would be

apparent if gross hyperactivity existed in these groups. We also looked at pre-CS freezing, which showed that 0.36 mg/kg increased freezing during the pre-CS time span (data not shown), which is counter to effects on contextual freezing and suggested decreased activity. Overall, these results suggest that JNK1 is necessary for the enhancement of contextual fear conditioning by nicotine and that the absence of JNK1 alters sensitivity to nicotine. In line with this possibility, the apparent decrease in freezing in high dose nicotine-treated JNK1 KO mice may indicate that JNK1 disruption alters nAChR function and that effect is revealed at higher doses of nicotine. Further, after backcrossing to C57BL/6J for 3 generations, JNK1 KO mice under vehicle treatment displayed normal levels of hippocampus-dependent learning in the present study, but nicotine treatment revealed the expected deficit associated with JNK1 disruption [20]. It is possible that depending on the specific strength and nature of the learning event, JNK1 and nAChR activity, may be recruited during the process of cellular memory formation. This is an area important for future experiments.

While the current experiments did not directly test whether a lack of JNK1 affected learning or memory recall of hippocampus-dependent contextual conditioning, other recent work has shown that nicotine affects learning [24] and not memory recall per se. In addition, previous work with the pan-JNK inhibitor SP600125 revealed that inhibiting JNK (isoforms 1-3) activation during consolidation was sufficient to block nicotine-enhanced contextual conditioning but JNK inhibition at recall had no effect [15]. Further, *Jnk1* gene expression was increased after learning (i.e., during consolidation), suggesting that consolidation is the critical stage where *Jnk1* makes its contribution to nicotine-enhanced contextual fear conditioning [15].

JNK1 has several downstream effectors in addition to the prototypical substrate c-Jun, such as c-Myc, Elk1, Atf-2, and p53 [29]. Nicotine is known to activate some downstream effectors of JNK1 in the hippocampus, such as Elk1 [23] and p53 [31], while it deactivates other effectors, including c-Myc [21]. In addition to its traditional nuclear substrates, JNK1 has several important cytosolic targets. JNK1 is implicated in trafficking of AMPA receptors to the membrane [30], which may affect neural excitability.

Additionally, JNK1 is important for PSD-95 phosphorylation [17] and microtubule associated protein activation and polymerization [5], which may be important for synapse modification. Thus, it is possible that stronger learning recruits JNK1 signaling within the nucleus to alter transcriptional activity or in the cytoplasm to alter synaptic structure and function and this could contribute to stronger learning. JNK1 has been shown to be important for the maintenance of axon fibers, but not their formation [5].

In summary, these results extend previous work to demonstrate that nicotine enhancement of hippocampus-dependent learning involves the recruitment of JNK1 signaling. This, along with prior work from our laboratory, suggests that the strength of learning may recruit different cell signaling cascades as the absence of JNK1 did not block learning but did prevent enhanced learning. Understanding how stronger memories are encoded has the potential to help target treatments for diseases associated with cognitive impairments. One potential limitation of KO studies is developmental effects. However, the fact that fear conditioning in saline-treated WT and KO mice was equivalent and a previous study using a non-specific inhibitor of JNK (1-3) in adult mice found similar results, lessens this concern. More studies are warranted to determine the upstream and downstream signaling molecules that work in concert with JNK1 to strengthen hippocampal memory formation.

Acknowledgments

This work was funded with grant support from the National Institute on Drug Abuse (T.J.G., DA017949). PTL and JWK were supported by NIH-NIDA Training Grant (DA007237).

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