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Remy E. Weinberger April 14, 2011

Role of Brain Derived Neurotrophic Factor and its receptor Tyrosine Kinase Receptor B in olfactory-dependent fear learning

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Abstract

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BDNF is known to play a role in neuronal survival and plasticity—especially during the processes of learning and memory. The olfactory system has been shown to undergo neurogenesis throughout adulthood; however it is unknown whether BDNF and its receptor TrkB are responsible for this process. Because of BDNF's role in plasticity, and it's increased presence in the olfactory bulb during an olfactory-dependent learning task, we chose to examine whether BDNF is responsible for the learning that occurs during an olfactory-cued learning paradigm. We found that mice were able to acquire learned fear to an olfactory stimulus, and that this effect was enhanced in mice that received a BDNF agonist, 7,8-DHF, prior to training. Furthermore, mice that were olfactory fear conditioned exhibited increased levels of BDNF mRNA at 2 and 4 hours post training, in addition to increased BDNF protein levels at 2 hours post training. Structural plasticity was also seen in M71-IRES-tauLacZ transgenic mice that were treated with vehicle prior to olfactory fear conditioning, but not in those that received the BDNF agonist, 7,8,3-THF. These data suggest that BDNF plays a role in olfactory-dependent fear learning; however, further studies must be conducted in order to determine whether BDNF is responsible for the structural plasticity that occurs in the glomeruli during this process.

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INTRODUCTION

Fear Conditioning

Pavlovian fear conditioning is a powerful tool to study learning and memory as well as anxiety disorders. During fear conditioning, a neutral stimulus, the conditioned stimulus (CS) (odor or tone), is paired with an aversive stimulus, the unconditioned stimulus (US) (foot shock). Following fear conditioning, presentation to just the conditioned cue without shock, elicits a fear response, which can be behaviorally measured as freezing (absence of movement) in rodents. These learned fear responses are highly dependent on the amygdala complex (Davis, 1992).

Animal models, especially mice and rats, have been shown to acquire, consolidate, and extinguish fear in a similar manner as humans, and have been essential to the understanding of fear and its neurocircuitry. Salient cues are necessary in order to effectively fear condition rodents (Rescorla & Wagner, 1972). Auditory and visual cues are both effective conditioned stimuli; however, olfactory stimuli are particularly salient cues that induce rapid and robust learning in rodents (Paschall & Davis, 2002; Jones et al., 2005). Furthermore, the olfactory system sends direct sensory projections to structures, such as the amygdala, known to be involved in the processing of aversive stimuli, providing an ideal model to study the neural mechanisms that occur as a result of conditioned fear (Pitkänen, Jolkkonen**,** & Kemppainen, 2000).

Cue-specific fear conditioning can be used to study the neural substrates involved in anxiety disorders precipitated by fearful memories. Individuals exposed to chemosensory signals associated with an anxious state have been shown to increase their own anxiety levels (Pause et al., 2009; Albrecht et al., 2010). Odors paired with illness have been shown

to increase fear-potentiated startle as well as analgesia (Richardson & McNally, 2002). Further, the olfactory system appears to be quite plastic, with some individuals able to 'train' their nose, such as in the perfume and wine industries, to increase sensitivity and range of olfactory sensation. The glomeruli in the olfactory bulb are responsible for receiving information from odorants and sending this information to the cortex for further processing (Kobayakawa et al., 2007). Although the mechanism by which specific odorants are processed in the brain has been identified (Buck et al., 2004), the mechanism by which certain odorants can be conditioned to elicit emotional responses is not yet fully understood.

Brain Derived Neurotrophic Factor and its receptor Tyrosine-Kinase receptor B

A candidate neural substrate for the process of olfactory-cued fear learning is brain derived neurotrophic factor (BDNF). Neurotrophic factors are responsible for regulating the survival and differentiation of specific populations of neurons (Levi-Montalcini, 1987). Additionally, they play a role in regulating synaptic plasticity (Thoenen, 1995). Plasticity can be defined in terms of both structural and functional changes. Functional changes can be activity-dependent, with simultaneous changes in biochemistry and morphology (Thoenen, 1995). In the central nervous system, neurotrophic factors are produced by neurons under physiological conditions (Lindholm et al., 1994). BDNF is an activity-dependent neurotrophic factor, whose mRNA production is up-regulated by glutamate via *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors and down-regulated by γ-aminobutyric acid (GABA) via $GABA_A$ receptors (Lindholm et al., 1994). The regulation of BDNF mRNA has also shown to be calcium-calmodulin-dependent (Zafra et al., 1992).

All neurotrophic factors originate as precursor polypeptides, which are then cleaved to form the individual mature active peptide (Thoenen, 1995). Each specific neurotrophic factor binds to an individual tyrosine kinase receptor, while they all share a common p75 receptor with low affinity (Heumann, 1994) (Figure 1a). BDNF binds with high affinity to its receptor tyrosine kinase receptor B (TrkB), activating a signaling cascade. In addition to binding to its full length TrkB receptor, BDNF can also bind to a truncated form of TrkB (Heumann, 1994). When BDNF binds the full-length TrkB receptor, it induces aggregation and autotyrosine-phosphorylation of the receptor causing the receptor membrane to recruit proteins involved in the intracellular signaling cascade (Kaplan & Miller, 2000) (Figure 1b). In one major pathway, MAPK is activated leading to fiber outgrowth (Kaplan & Miller, 2000). Other signaling pathways activated by TrkB activation include the PI3K and phospholipase C-γ1 pathways (Kaplan & Miller, 2000). The signaling cascade leads to the activation of the c-*fos* promoter by phosphorylated Elk/SRF and CREB transcription factor complexes (Heumann, 1994). The activation of c-*fos* is shown to be involved in neuronal excitability and survival (Zhang et al., 2002). Additionally, c-*fos* regulates the expression of BDNF (Zhang et al., 2002).

7,8-Dihydroxyflavone (7,8-DHF) has recently been identified as a high-affinity TrkB agonist (even greater affinity than BDNF) that mimics the effects of BDNF by triggering the receptor dimerization and autophosphorylation of TrkB, along with activating the downstream signaling cascade (Jang et al., 2010). Moreover, 7,8-DHF has been shown to prevent neurons from undergoing apoptosis (Jang et al., 2010) and to enhance auditory fear and extinction learning (Andero et al., 2011). The use of 7,8-DHF in studies of

olfactory-cued fear conditioning has the potential to enhance the understanding of BDNF's role in cue-specific fear learning.

BDNF/TrkB in Learning and Memory

The synaptic plasticity that occurs during learning and memory formation is an activity-dependent process (Tyler et al., 2002). BDNF is a potential modulator of synaptic plasticity because its expression is regulated by neuronal activity and bioelectrical activity (Zafra et al., 1990; Zafra et al., 1991). Additionally, hippocampal BDNF mRNA is modulated by neurotransmitters and neuromodulatory systems involved in hippocampal-dependent learning and memory (Tyler et al., 2002). *In vivo* BDNF mRNA levels have been shown to increase following increased activity and exposure to enriched environments—both shown to be involved in learning and memory (Tyler et al., 2002). BDNF mRNA has also been shown to increase following learning (Kesslak et al., 1998). Furthermore, mice without hippocampal BDNF were unable to learn hippocampal-dependent tasks (Heldt et al., 2007).

Previous studies from our lab have shown that BDNF plays a critical role in learning and memory in other areas in addition to the hippocampus. A study by Rattiner et al. showed that BDNF expression in the basolateral amygdala is temporally regulated during the consolidation period following cued fear-conditioning, while the TrkB receptor concurrently undergoes increased phosphorylation, indicating potential activation of the receptor by BDNF (Rattiner et al., 2004). Mice with a truncated form of TrkB are unable to acquire cued fear memories, unlike mice with the full-length receptor; thus, showing the essential role of TrkB in the amygdala-dependent acquisition of fear memories (Rattiner et al., 2004). Additionally, a truncated form of TrkB blocks the extinction of cued fear

(Chhatwal et al., 2006). The ability to consolidate fear memories is lost in mice with a conditional BDNF knockout (Figure 2); however, the administration of the TrkB agonist 7,8-DHF restores the ability of these mice to consolidate new learned fear memories (Choi et al., 2010).

Olfactory System

Mammals can sense an enormous number and variety of odorant chemicals as having different odors. It is estimated that humans, for example, can detect from 10,000 to over 100,000 different volatile compounds (Buck, 2004). The ability of mammals to distinguish between a vast amount of odors lies in the ability of distinct odorants to activate a discrete population of olfactory sensory neurons (Buck, 2004). Each sensory neuron in the olfactory epithelium of the nose expresses a distinct receptor type (Ressler et al., 1993; Vassar et al., 1993). Within the epithelium there are four zones that each contain non-overlapping sets of olfactory receptor genes, which are expressed in a small percentage of neurons in that zone (Buck, 2004). These sensory neurons then project to the glomeruli of the olfactory bulb, by way of the olfactory nerve, and converge on only one to four of the 2000 glomeruli in the olfactory bulb; thus, preserving the spatial organization of receptors in the nose to form a topographic sensory map (Mombaerts et al., 1996) (Figure 3a). The axons of the olfactory sensory neurons synapse with mitral and tufted cells in the glomeruli of the olfactory bulb, and these cells send axons to the olfactory cortex where cortical neurons receive and integrate input from a combination of multiple different olfactory receptors (Buck, 2004) (Figure 3b). The piriform cortex is the primary area of the olfactory cortex responsible for processing olfactory information (Buck, 2004). The spatial

organization present in the nose and bulb is lost in the piriform cortex, where instead, unique ensembles of neurons are activated in response to a specific odorant (Zou et al., 2001). Olfactory information is also transmitted to subcortical structures, like the amygdala, that can further process the emotional and motivational aspects associated with the odor (Buck, 2004). The amygdala has been shown to play a critical role in the acquisition and expression of fear conditioned by an olfactory stimulus (Otto et al., 2000).

Olfactory System Plasticity

The olfactory system has been shown to undergo learning-induced changes including long-term potentiation (Ennis et al., 1998), increased pyramidal neuron spine density (Knafo et al., 2001), and enhanced Fos expression in the primary olfactory and limbic pathways (Funk and Amir, 2000). Furthermore, kainic acid-induced seizure activity, which increases neuronal activity, causes a dramatic increase in BDNF in the olfactory bulb and piriform cortex (Katoh-Semba et al., 1999). Accordingly, BDNF deficits in the olfactory bulb result in a decrease in dendritic spine density (Nanobashvili et al., 2005). BDNF has also been shown to play a role in the differentiation and survival of regenerating cells in the olfactory bulb (Benraiss et al., 2001).

The olfactory system is an intriguing area to study plasticity because it continues to undergo neurogenesis throughout adulthood (Jones et al., 2008). A study conducted by Jones and colleagues used an olfactory-cued learning paradigm to show that BDNF is dynamically regulated in different areas of the adult olfactory system. BDNF mRNA levels in the mitral cell layer of the main olfactory bulb, the anterior and posterior piriform cortices, and the basolateral amygdala were measured using *in-situ* hybridization (Jones et

al., 2007). Levels of BDNF were measured at 2 hours following fear conditioning, since maximum BDNF mRNA induction occurs at this time point following fear conditioning within the amygdala (Rattiner et al., 2004). In mice that received odor-shock pairings, BDNF was significantly increased in the bulb, anterior piriform, posterior piriform, and the basolateral amygdala; however, in mice that received odor alone, BDNF only increased in the bulb and anterior piriform cortex (Jones et al., 2007). These results suggest that the olfactory bulb and anterior piriform cortex are involved with odor identification, while the posterior piriform cortex and the basolateral amygdala play a role in associative learning (Jones et al., 2007). Additionally, the olfactory receptor cells contain TrkB mRNA and olfactory axonal membranes contain TrkB immunoreactivity, suggesting that BDNF plays a role in olfactory signaling or in trophic interactions between the olfactory bulb and olfactory sensory neurons (Hasegawa et al. 2008).

Structural changes in the olfactory system also occur during olfactory learning. The ability to discriminate between previously indiscriminable odorants is enhanced when the odor is associated with an aversive stimulus (Li et al., 2008). The ability to perceive these differences is accompanied by plasticity in the piriform cortex (Li et al., 2008). Another study showed that there is structural plasticity in the olfactory sensory neurons that project to the glomeruli in the olfactory bulb with odor learning (Jones et al., 2008). This study utilized the M71–IRES–tauLacZ transgenic mouse to visualize changes in a specific population of olfactory sensory neurons during olfactory-cued fear conditioning (Vassalli et al., 2002) (Figure 4). These mice contain a *Lac*Z reporter, which encodes β-galactosidase, neurons that express the M71 odorant receptor, enabling visualization of changes in density and axon projections of the M71 glomeruli. Furthermore, the odorant

acetophenone is a specific agonist for the M71 receptor; thus, providing a stimulus that discretely activates the specific population of olfactory sensory neurons of interest (Bozza et al., 2002). Jones and colleagues found that mice that were trained to associate acetophenone with an aversive foot shock had significantly increased axon density and glomerular size as compared to their littermates that did not receive this training or those only exposed to the odor alone. These data demonstrate that structural plasticity occurs in the olfactory system as a function of environmental experiences in adults (Jones et al., 2008). The neural mechanism underlying these structural changes has yet to be determined. However, the induction of BDNF mRNA following olfactory-cued fear conditioning suggests it as a strong candidate for a neural substrate involved in the learning-induced structural changes exhibited in the bulb.

Hypothesis & Aims

Based on the data showing that structural changes in the olfactory system occur after olfactory-cued fear conditioning and that BDNF is present in the olfactory system during this process, we hypothesize that the BDNF signaling system is a critical mechanism underlying the learning that occurs during olfactory-cued fear conditioning. We will test this hypothesis with the following specific aims: (1) Determine if the novel TrkB agonist 7,8-DHF enhances olfactory-cued fear learning, (2) Determine using a more quantitative method of mRNA determination, rtPCR, that BDNF mRNA is increased following fear learning in the olfactory bulb, (3) Determine whether BDNF protein in the olfactory bulbs increases with olfactory-cued fear conditioning in wild type mice.

METHODS AND MATERIALS

Subjects:

Adult (6-8 weeks of age) male C57BL/6J mice weighing 20-30 g (Jackson Laboratories, Bar Harbor, ME, USA) were used for all experiments, except adult male M71–IRES–tauLacZ transgenic mice (Vassalli et al., 2002) maintained in mixed 129/Sv x C57BL/6 background (Jackson Laboratories) were used in the 7,8,3-THF pilot drug study and neocortical BDNF knockout mice (Choi et al., 2010) used in the pilot ELISA assay. Cortex-specific BDNF knockout mice (Cre+) were created when the coding sequence for Cre recombinase was placed downstream of a 3-kb CCK promoter and the cortex-specific Cre line was then crossed to floxed BDNF mice. Mice that did not express Cre recombinase (Cre-) that were then crossed to floxed BDNF were also used. Animals were housed in standard group cages (four per cage) and were given ad libitum access to food and water. All experiments were performed during the light portion of a 12-hour light/dark cycle. All experiments were approved by Emory University Institutional Review Board following Institutional Animal Care and Use Committee (IACUC) standard with accordance to the Yerkes Primate Research Center regulations.

Groups:

Mice were grouped depending on which aim they were designated toward. Wild type mice used in aim 1 were injected with 7,8-DHF (n=8) or vehicle (n=11) prior to olfactory-cued fear conditioning and were tested for freezing levels 24 hours later. During the week prior to training, these mice received three consecutive days of handling, including injection with vehicle, followed by one day of habituation in the fear conditioning

chambers. The wild type mice used in aim 2 were divided into 6 groups: home cage controls (n=8), mice that received exposure to odor only (n=8), and mice that received olfactory-cued fear conditioning with odor/shock pairings that were sacrificed 0 minutes (n=8), 30 minutes (n=8), 2 hours (n=8), and 4 hours (n=8) post-training. Wild type mice used in aim 3 received olfactory-cued fear conditioning (n=12) or no training (n=12) and were sacrificed 2 hours post-training. Additionally, M71–IRES–tauLacZ mice were used to expand upon the findings that olfactory-cued fear conditioning leads to an increase in glomerular area. Prior to training, these mice received either 7,8,3-THF, a BDNF agonist, (n=12) or vehicle (n=12). Home cage control mice that received no fear training were also analyzed (n=12). These M71–IRES–tauLacZ transgenic mice were then perfused 3 weeks following training and their bulbs were stained for LacZ expression. Neocortical BDNF knockout mice (Cre+) (n=12) and littermate controls (Cre-) (n=12) were used to determine the sensitivity of the ELISA assay used in aim 3. These mice received tone/shock cued fear conditioning and were sacrificed 30 minutes after training.

Drugs:

7,8-dihydroxflavone (7,8-DHF) and 7,8,3-trihydroxyflavone (7,8,3-THF) were dosed systemically (i.p.) at a 5-mg/kg dose in 17% DMSO in PBS. Vehicle was 17% DMSO in PBS.

Fear Conditioning Apparatus:

Fear training and testing sessions were conducted in four identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA). Each consisted of a nonrestrictive Plexiglas cylinder (5.5 cm diameter, 13 cm long) mounted on a Plexiglas platform that was

located in a ventilated, sound-attenuating chamber. The floor of each cylinder was a cradle-shaped grid that contained seven 3.0-mm diameter stainless steel bars spaced 1 cm apart, through which shock could be delivered. Cylinder movements were detected by a piezoelectric accelerometer mounted under each platform and were digitized and stored by an interfacing computer assembly as voltage output sampled each millisecond.

The foot shock US was generated by a programmable animal shocker (San Diego Instruments) located outside the isolation chambers and was delivered through the cage floor bars.

For olfactory fear conditioning, the conditioned stimulus odor was delivered to chambers in a manner similar to that described previously (Paschall & Davis, 2002; Jones et al., 2005). Briefly, a compressed air tank with a pressure regulator and flow meter delivered a constant flow rate of 40 L/min. The flow meter output was split with a Y-connector to create two separate delivery lines: a clean, odor-free line and an odor-delivery line that was connected to a solenoid valve controlled by a computer running the SR-Lab software. PharMed Tygon tubing (3.2 mm id; Saint-Gobain, Akron, OH) was used to form delivery lines because of its low permeability to vapors. When the valve opened, air flowed through the odor-delivery line into a sealed jar containing the dissolved odorant. Tubing from the jar merged with the odor-free line to form a single 80-cm delivery line that fed into the front of the Plexiglas cylinder. When the valve closed, air flowed thorough the odor-free line only. Opening and closing of the solenoid valve did not produce any difference in rate of air flow to the cylinder. Backflow was prevented by one-way valves. The odor was rapidly removed from the back of the cylinder via an exhaust hose feeding into the room's ventilation fan.

Fear Conditioning Procedures:

Training- For olfactory-cued fear conditioning, a mouse was placed in a chamber and exposed to ten trials of odor/shock pairings, where the odor was a 10 sec exposure to 10% acetophenone in propylene glycol, co-terminating with a 250ms 0.4 mA foot shock. The first pairing was presented after 5 minutes in the chamber and the intertrial interval was 5 minutes. For tone/shock fear conditioning, a mouse was placed in a chamber and exposed to ten trials of tone/shock pairings, consisting of a 30s 6kHz tone at approximately 80dB tone co-terminating with a 500ms 1 mA footshock. The first pairing was presented after 5 minutes in the chamber and the intertrial interval was 5 minutes.

Testing- Testing was conducted 24-hours after fear training. Mice were placed in chambers for 50 minutes and their activity without stimulus presentation was measured 36 times over a 10 second period and their activity during presentation of the odor alone was measured 10 times over a 10 second period. Freezing was interpreted as the difference between activity alone and activity during odor presentation, where the lack movement/activity was indicative of freezing.

ELISA Assay:

Tissue was immediately frozen on dry ice following extraction. One-millimeter micropunches of the hippocampus, prefrontal cortex, and amygdala were taken from the Cre+ and Cre- mice, while both bulbs were extracted from the wild-type mice that received olfactory-cued fear conditioning. Tissue was processed using a lysis buffer (137mM NaCl, 20mM Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1mM PMSF, 10µg/ml aprotinin, 1µg/ml

leupeptin, 0.5mM sodium vanadate). Samples were diluted to 1mg/ml with lysis buffer. Samples were then treated with 1.0M HCl for 20 minutes at a pH of 3.0 and then neutralized to a pH of 7.8 using 1.0M NaOH. A 96-well Corning Costar® ELISA plate was coated with 100ul of 10µl Anti-BDNF mAb (Promega® BDNF ImmunoAssay System) diluted in 9.99ml carbonate coating buffer (0.025M sodium bicarbonate, 0.025M sodium carbonate, adjusted to a pH of 9.7) and stored at 4°C overnight. The wells were washed once with TBST wash buffer (20mM Tris-HCl (pH 7.6), 150mM NaCl, 0.05% (v/v) Tween® 20) and then blocked with 100ul of 1x Block and Sample Buffer (Promega® BDNF ImmunoAssay System) in each well at room temperature for 1 hour. TBST buffer was used to wash the wells once prior to the addition of 100ul of tissue sample to each well. A standard curve of BDNF standard (Promega® BDNF ImmunoAssay System) was also conducted with concentrations ranging from 500-0 pg/ml. The tissue and BDNF standard was left to shake at 400rpm overnight at 4°C. The wells were washed 5 times with TBST wash buffer and 100ul of 20µl of Anti-Human BDNF pAb (Promega® BDNF ImmunoAssay System) diluted in 9.98ml of Block & Sample 1X Buffer was then added to each well. The plate was then shaken at room temperature for 2 hours at 400rpm. TBST was buffer was then used to wash the plate 5 times and 100ul of 50µl of Anti-IgY HRP Conjugate (Promega® BDNF ImmunoAssay System) diluted in 9.95ml of Block & Sample 1X Buffer was then added to each well. The plate was shaken at room temperature for one hour. The wells were then washed 5 times with TBST wash buffer and 100ul of TMB One Solution (Promega® BDNF ImmunoAssay System) was added to each well. The plate was shaken at 400rpm for 10 minutes and then 1N HCl was added to each well. The absorbance at 450nm was immediately read using a plate reader (Microplate Managing Systems).

RNA Extraction:

Brains were extracted from animals and were immediately frozen on dry ice. One-millimeter micropunches were taken from the olfactory bulbs. The Qiagen RNeasy Kit was used to extract the RNA from the micropunches. These punches were then homogenized in 350 ul of Buffer RLT (Qiagen) using a sonicator. 350 ul of 70% ethanol was added to each sample. 700 ul of the sample was transferred to an RNeasy spin column (Qiagen) and centrifuged for 15 s at 10,000 rpm. 700 ul of Buffer RW1 (Qiagen) was added followed by centrifugation at 15 s at 10,000 rpm. 500 ul of Buffer RPE (Qiagen) was added followed by centrifugation for 15s at 10,000 rpm. This step was repeated, but centrifuagtion time was increased to 2 minutes at 10,000 rpm. Finally, 50 ul of RNase-free water (Qiagen) was added to the spin column, followed by centrifugation for 1 minute at 10,000 rpm, and the filtrate was then collected. RNA concentrations (ng/ul) were quantified using a nanodrop apparatus.

cDNA Synthesis:

cDNA was synthesized from the RNA of the bulbs using the RT^2 First Strand Kit (Qiagen). 80 ng of RNA from each sample was mixed with 5X Genomic DNA Elimination Buffer (Qiagen) and incubated at 42 degrees C for 5 minutes. After being briefly chilled on ice, 2.5 ul of RT Cocktail (5X RT Buffer 3, Primer and External Control Mix, RT Enzyme Mix 3—Qiagen) was added to each Genomic DNA Elimination Mixture. The samples were then incubated at 42 degrees C for exactly 15 minutes and the reaction was immediately stopped by heating to 95 degrees C for 5 minutes. cDNA (ng/ul) was quantified using a

nanodrop apparatus.

cDNA was synthesized from the RNA of the posterior piriform cortex and the basolateral amygdala using the High-Capactiy cDNA Reverse Transcription Kit (Applied Biosystems). 10 ul of 2X RT master mix (10X RT Buffer, 25X dNTP Mix (100mM), 10X RT Random Primers, Multiscribe™ Reverse Transcriptase—Applied Biosystems) was added to 10 ul of each RNA sample. This mixture was then placed in the thermal cycler at 25 degrees C for 10 minutes, 37 degrees C for 120 minutes, and 85 degrees C for 5 minutes. cDNA (ng/ul) was quantified using a nanodrop apparatus.

Real-Time PCR:

Master Mix consisted of 10 ul of TaqMan® 2X PCR Mix (Applied Biosystems), 1 ul of either BDNF or GAPDH primers, and RNase-free water. 18 ul of master mix was combined with 2 ul of the cDNA normalized to the lowest concentration (ng/ul). This mixture was then run through the RT-PCR program (Applied Biosystems 7500 Fast) at the following cycling conditions: 2 minutes at 50 degrees C, 10 minutes at 95 degrees C, followed by 40 repetitions of 15 seconds at 95 degrees C and 1 minute at 60 degrees C.

Beta-galactosidase staining:

Three weeks following the onset of odor shock fear conditioning, the mice used in aim 3 were perfused with 4% paraformaldehyde in PBS. Brains were stained for b-gal, using 45 mg of X-gal (1 mg/ml) dissolved in 600 ul of DMSO and 45 ml of a solution of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl in 1 M PBS, and incubated overnight at 37°C.

M71 olfactory glomerular quantification:

Using a microscope-mounted digital camera, high-resolution images were captured at 40X magnification of the M71-positive glomeruli. Images were converted to grayscale and equalized for background brightness. X-gal-labeled glomerular area was quantified as pixels, less than a set threshold gray level of 150 (optimized for axon vs background).

Statistical analyses:

Data were analyzed with T-tests and 2-way Analysis of Variance (ANOVAs). Significance was set at alpha=0.05

RESULTS

To determine if 7,8 DHF can augment olfactory fear learning (aim 1), mice were first test to determine the average amount of freezing due to olfactory conditioning. Mice were olfactory fear conditioned and then tested to the odor CS alone 24 hours later. They showed a significant decrease in activity when exposed to the odor alone when compared to their activity during the absence of the odor ($p<0.01$) (Figure 5). This decrease in activity is the same as an increase in freezing—a behavioral measure of learned fear. Because the mice exhibited an increase in freezing, this training protocol is sufficient to induce fear; thus, supporting its use in the following study where the BDNF agonist, 7,8-DHF, was administered.

In aim 1, we then tested the effect of the BDNF agonist, 7,8-DHF, on the ability of mice to learn olfactory-dependent fear memories. Mice received intraperitoneal injections of 7,8-DHF and vehicle immediately before undergoing olfactory fear conditioning. There was a significant effect for both the drug administered and whether the odor was present during the activity measurement; however, there was no significant interaction between these two variables. Mice showed significantly reduced activity when exposed to the odor CS alone 24-hours later ($p<0.05$) (Figure 6). This reduction in activity is synonymous with an increase in freezing. Furthermore, the mice given 7,8-DHF showed a significantly greater reduction in activity than those given vehicle ($p<0.05$) (Figure 6). The increase in freezing shown in the group that received 7,8-DHF demonstrates a possible role for BDNF in this learning process.

Since we found an effect of BDNF in regulating our olfactory dependent fear conditioning, in our next experiment, we investigated whether there would be differences

in BDNF expression following fear conditioning. In aim 2 we determined if BDNF mRNA increases with olfactory-cued fear conditioning. Mice were olfactory fear conditioned and then sacrificed 0 minutes, 30 minutes, 2 hours, and 4 hours following training. Their BDNF mRNA expression was quantified using rtPCR (Figure 7) and compared to mice that did not receive training and mice that were exposed to the odor only. An increase in BDNF mRNA levels was shown at 2 and 4 hours post training (Figure 8). These data are preliminary, and not all groups successfully reached the threshold for cT value determination after 40 cycles of rtPCR; therefore, we were unable to perform statistical analyses on this data. The increase in BDNF suggests plasticity is occurring during olfactory fear learning, with a robust BDNF induction around 2-4 hours post conditioning in the olfactory bulbs.

In order to see if the changes in BDNF mRNA lead to changes in BDNF protein, BDNF protein levels were examined using an ELISA assay. Prior to examining BDNF protein levels in the bulbs, we looked at BDNF expression in the hippocampus, prefrontal cortex, and the amygdala—areas known to express BDNF, to demonstrate that the ELISA is able to detect differences in BDNF levels. Neocortical BDNF knockout mice were fear conditioned to a tone CS. BDNF protein levels were determined from a standard curve of values obtained from an ELISA assay (Figure 9a). Neocortical BDNF knockout mice (Cre+) expressed significantly less BDNF protein in the hippocampus than mice expressing BDNF (Cre-) ($p<0.01$). The BDNF knockouts also had a robust trend of less BDNF protein in the prefrontal cortex but no differences were found in the basolateral amygdala (Figure 9b). The ability of this assay to detect differences in BDNF between different tissue samples validates its usage in examining BDNF levels in the olfactory bulbs.

In order to look at BDNF protein expression in the olfactory bulb following olfactory-cued fear learning, mice were olfactory fear conditioned and their bulbs were extracted for processing 2 hours following training. BDNF protein levels in the olfactory bulb were determined from a standard curve of values obtained from an ELISA assay (Figure 10a). Olfactory bulbs from mice that underwent olfactory fear conditioning contained significantly greater BDNF protein ($p=0.05$) than bulbs from mice that did not receive any training (Figure 10b). Increases in BDNF protein indicates that plasticity could be occurring in the olfactory bulb during this learning process.

The M71 transgenic mouse model was used as a tool to examine structural plasticity in the olfactory bulb. Structural changes in M71 glomeruli were examined after olfactory fear conditioning to determine if BDNF plays a role is this structural plasticity. Mice were injected intraperitoneally with 7,8,3-THF or vehicle prior to olfactory fear conditioning. These mice and control mice were perfused three weeks following training, when structural plasticity was previously shown to occur, and stained for LacZ expression (Figure 11). Both the 7,8,3-THF and the vehicle treated mice did not show any significant differences in dorsal glomerular area with each other or with the dorsal control mice; however, the medial glomeruli of the mice treated with vehicle had significantly greater area than the glomeruli of the mice treated with THF and the medial controls ($p<0.05$) (Figure 12). The increase in glomerular area in the vehicle group suggests that plasticity is occurring during this learning process, consistent with data from Jones et al.; however, the 7,8,3-THF group does not confirm that BDNF and TrkB are responsible for this structural change.

DISCUSSION

Our data provides evidence supporting the hypothesis that BDNF and its receptor TrkB play a critical role in olfactory-dependent fear learning. Behavioral studies showed mice exhibited a decrease in activity, or an increase in freezing, when presented with an aversive odor. This response is enhanced in mice that received the TrkB agonist, 7,8-DHF, prior to training. Furthermore, rtPCR analysis of BDNF mRNA levels following olfactory fear conditioning showed an increase in BDNF mRNA at 2 and 4 hours post training. In agreement with the mRNA findings, BDNF protein levels examined by an ELISA assay showed a significant increase in BDNF protein at 2 hours post-training. The ELISA assay for this protein study also showed significant differences in BDNF levels in conditional BDNF knockout mice; thus, proving its sensitivity to detect BDNF differences in the olfactory bulb. Finally, the study that examined M71 glomeruli 3 weeks post-training showed a robust increase in the glomerular area of mice that received vehicle prior to olfactory-cued fear conditioning showing learning dependent structural plasticity consistent with data from Jones et al. (Jones et al., 2008); however, mice that received 7,8,3-THF did not show a significant increase in glomerular area as expected. Although 7,8,3-THF theoretically binds to TrkB with a higher affinity than 7,8-DHF, it could have caused other adverse systemic effects that could have blunted the olfactory-dependent structural plasticity because it was injected intraperitoneally.

In summary, our data demonstrated an increase in TrkB induced olfactory-dependent fear learning, complemented by significant increases in both BDNF mRNA and BDNF protein; consequently, supporting the hypothesis that BDNF and its receptor TrkB play a critical role in olfactory-dependent fear learning. Additionally, the results from the

pilot 7,8,3-THF study are preliminary and there is still no convincing evidence to prove that BDNF and TrkB are not regulators of the learning-dependent structural plasticity that was exhibited in vehicle treated mice.

The ability of mice to acquire conditioned fear, exhibited by an increase in freezing levels, to an olfactory stimulus is consistent with previous data from our lab (Jones et al., 2005) and previous studies done in rats (Otto, Cousens, & Rajewski, 1997; Paschall & Davis, 2002). Furthermore, the greater freezing effect seen in mice treated with 7,8-DHF is consistent with other data in our lab that demonstrated that TrkB agonist enhances emotional learning (Choi et al., 2010; Andero et al., 2011). These data suggest that TrkB activation does enhance olfactory dependent fear conditioning, however, it is unclear with systemic injections, where TrkB is targeting. Based on previous work in our laboratory, it is possible that TrkB in the basolateral amygdala may one potential target (Rattiner et al 2004), or it could be within the olfactory system in the olfactory bulbs or the piriform cortex. Future studies may address this by utilizing local infusions of our TrkB agonist, 7,8-DHF, into the bulbs or amygdala during olfactory fear conditioning.

The next experiments investigated the induction of BDNF expression specifically in the olfactory bulbs following olfactory fear conditioning. Our mRNA data is consistent with previous *in-situ* hybridization studies from our lab . The robust increase in mRNA at 2 and 4 hours post-training is consistent with the finding that the greatest induction of BDNF mRNA is found at 2 hours post-training as well as the finding that BDNF mRNA increases in the olfactory bulb 2 hours following olfactory-cued fear conditioning (Jones et al., 2007). The increase observed in BDNF protein levels at 2 hours post-training is also consistent with this data. These data indicate that BDNF expression plays an important role in the

olfactory bulbs following fear conditioning to a specific odor. With the olfactory system being a select region of the brain that continues to have ongoing neurogenesis, BDNF is likely involved in trophic activity and plasticity in the olfactory system that promotes either recruitment of olfactory epithelium neurons specific to the odor or the increase in branching and density of axons making up the glomeruli in the bulbs.

The final aim investigated whether olfactory dependent plasticity occurs with TrkB signaling. The results observed in vehicle treated M71 mice are consistent with previous studies in our lab, in which M71 mice that underwent olfactory-cued fear conditioning with acetophenone odor showed a robust increase is glomerular area (Jones et al., 2008). Because the pilot study with 7,8,3-THF did not show an increase in glomerular area, further studies must be conducted to investigate the mechanism behind these changes, and whether BDNF and TrkB are involved. 7,8-DHF has been more widely studied than 7,8,3-THF. Although it has a slightly lower affinity for BDNF than 7,8,3-THF, 7,8-DHF has been shown to cross the blood-brain barrier and activate TrkB receptors in the brain (Jang et al., 2010) and enhance emotional learning (Choi et al., 2010; Andero et al., 2011); thus, 7,8-DHF seems to be a more promising drug for future glomeruli studies. Alternatively, systemic administration of 7,8,3-THF may have been too strong of a dose and could have lead to excessive or adverse effects of TrkB activation that disrupted the learning dependent plasticity as indicated by only a trend in increase of glomerular area. Thus, additional experiments need to optimize the use of 7,8,3-THF because our results demonstrate that enhanced TrkB activation, by 7,8,3-THF, does not necessarily equate to greater enhanced learning and/or learning dependent plasticity of the olfactory system.

Further experiments need to be conducted in order to explore the induction of BDNF mRNA and protein following olfactory-cued fear conditioning in other brain regions known to show differences in BDNF expression following fear learning. The previous *in-situ* hybridization studies conducted in our lab not only examined mRNA in the olfactory bulb, but also in other areas of the olfactory pathways that included the anterior and piriform cortices and the basolateral amygdala (Jones et al., 2007). RtPCR analysis should be used to quantify BDNF mRNA in theses regions at the same time points as were measured in the bulb. An ELISA assay should also be conducted to measure protein in these regions as well. The previous *in-situ* study showed increases in BDNF mRNA in the olfactory bulb and anterior piriform cortex that was not dependent on odor-shock association; however, association was required for BDNF mRNA induction in the posterior piriform cortex and the basolateral amygdala (Jones et al, 2007). Additionally, changes in mitral cell receptive fields (Fletcher & Wilson, 2003) and the anterior piriform cortex (Wilson, 2003) occur with increased odor exposure, regardless of odor-shock association. Because these areas differ, it is essential to use quantitative PCR and BDNF protein analyses to help determine where and when neuroplasticity is occurring in the olfactory system in order to uncover the mechanism behind this learning process and determine targeted treatments for anxiety disorders affected by this mechanism.

Understanding the mechanisms involved in olfactory-dependent learning and memory is essential in order to further our understanding of potential targets to treat debilitating disorders impacted by the learning and memory processes involved in fear—particularly post-traumatic stress disorder (PTSD). Olfaction has been shown to both precipitate PTSD symptoms and play a role in the re-experiencing of symptoms (Vermetten

& Bremner, 2003). Odor memories are long-lasting and independent of other types of memory (Schab, 1991); thus, they may be more difficult to extinguish in patients with PTSD. Therapies have evolved to treat PTSD with olfactory components. Hypnotheraputic olfactory conditioning uses the ability of humans to associate pleasurable odors with a sense of control and calm, while placing them in the context of the fearful stimulus, in order to allow them to associate the safe odor with the context instead of the fear-eliciting odor (Abramowitz & Lichtenberg, 2010). Although this therapy has been shown effective in some individuals, other therapies, including pharmacological treatments, should also be considered. Answers concerning the learning, processing, and extinction of olfactory fear memories in patients with PTSD will provide pivotal insight into furthering treatments for PTSD and other anxiety disorders.

In summary, our data suggests that BDNF and its TrkB receptor play important roles in learning dependent plasticity that occurs in the olfactory system during olfactory-cued fear conditioning. This mechanism and others may provide further understanding of how fear learning is modulated and future discovery of potential targets for treating the debilitating fearful memories experienced by patients with PTSD.

FIGURES

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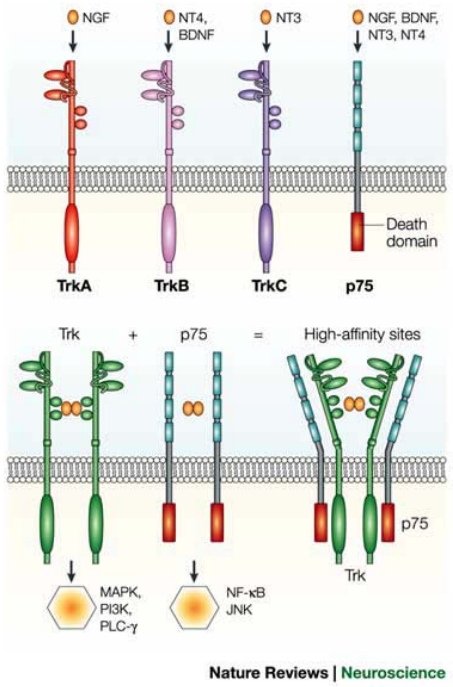

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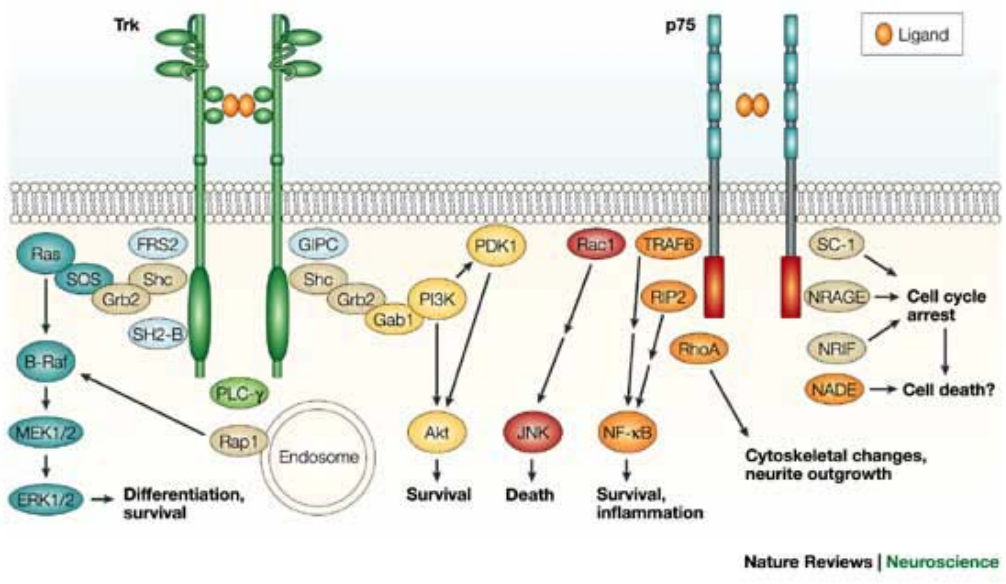

Figure 1. *Overview of neurotrophin signaling systems (Chao, 2003)*
Different neurotrophins bind selectively to different Trk receptors; however, all neurotrophins share affinity for the p75 receptor (Figure 1a). BDNF binds with high affinity to TrkB. Upon binding, Trk receptors dimerize, activating the extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLC-γ) pathways that mediate survival and differentiation (Figure 1b).

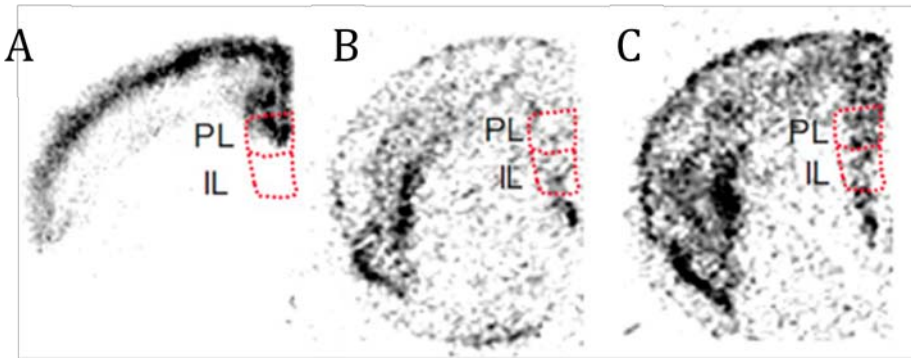

Figure 2. *Neocortical BDNF knockout mouse (Adapted from Choi et al., 2010).* Neocortical BDNF knockout mice (Cre+) were generated by crossing mice that expressed Cre recombinase in the PL and neocortex, but not the IL, (Figure 2a) with homozygous BDNF-floxed mice. In BDNF knockout mice, BDNF expression is deleted in the areas of the cortex where Cre is present (Figure 2b), but not in the littermate controls that do not express Cre (Figure 2c). These mice were used to test the sensitivity of the BDNF ELISA immunoassay.

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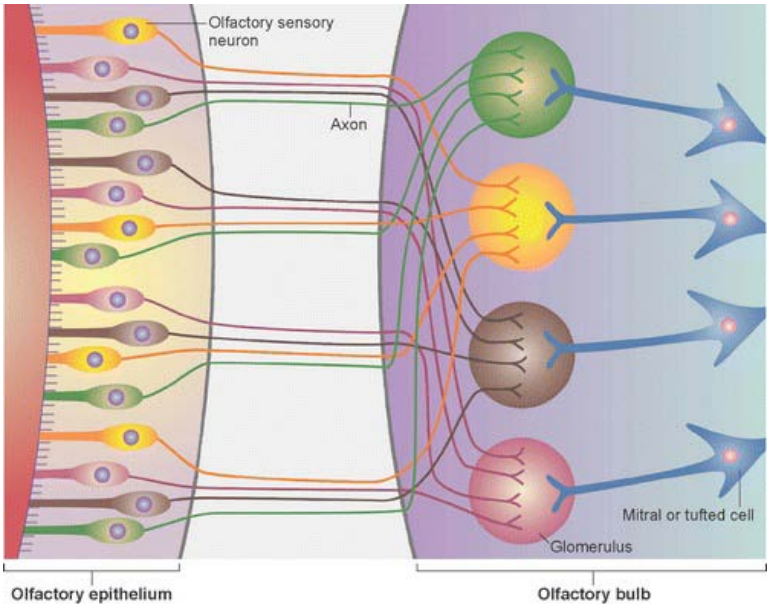

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Annu. Rev. Cell Dev. Biol. 22:713–37

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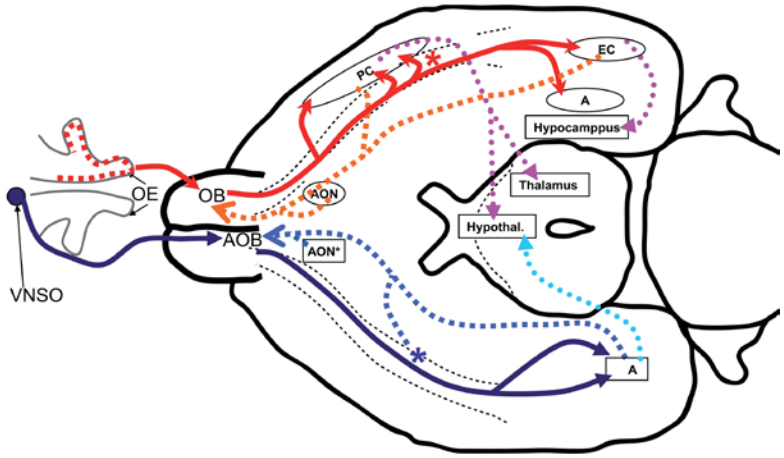

Figure 3. *Overview of the olfactory system(Dulac and Torello, 2003 (3a); de Castro, 2009 (3b))*

In the main olfactory system (Figure 3a), four different populations of olfactory sensory neurons (OSNs) that each express a different olfactory receptor are depicted in green, orange, brown, and red. The OSNs are scattered throughout the olfactory epithelium, but the axons of the OSNs expressing the same receptor converge at the same glomeruli in the olfactory bulb where they synapse with the dendrites of the second-order neurons, mitral or tufted cells. The axons of the mitral and tufted cells project their axons to the olfactory cortex where odor processing occurs. A schematic diagram of the connections made in the main olfactory system, depicted in red, shows the pathway of olfactory information from the olfactory epithelium (OE), to the olfactory bulb (OB), where the axons of the projection neurons project to the piriform cortex (PC), entorhinal cortex (EC), and the amygdala (A) (Figure 3b).

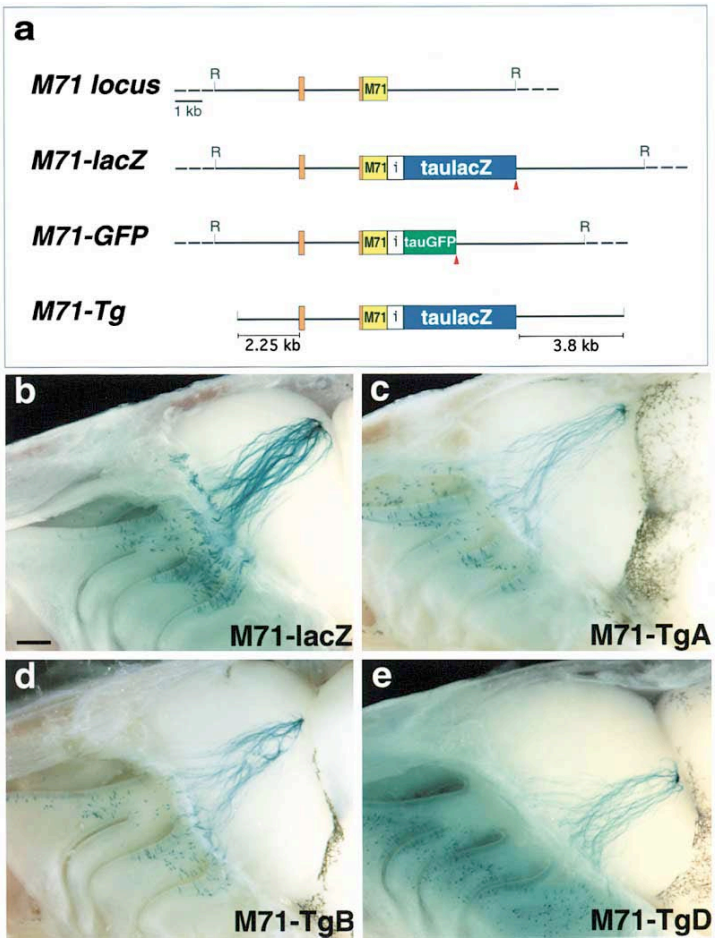

Figure 4. *M71–IRES–tauLacZ transgenic mouse (Adapted from Vassalli et al., 2002)*
The structure, targeted mutagenesis, and trangenesis at the M71 locus is shown in Figure 4a. Cre-mediated recombination was used to generate the *M71–IRES–tauLacZ* allele and the *M71–IRES–tauGFP* allele. *M71-Tg* represents the transgene allele. Figures b-e show the labeled OSN cell bodies in the *M71-lacZ* heterozygous mouse (Figure 4b) and hemizygous mice of various transgenic lines (Figure 4c-e). An *M71–IRES–tauLacZ* transgenic mouse line was used in the pilot study that examined M71 glomerular area.

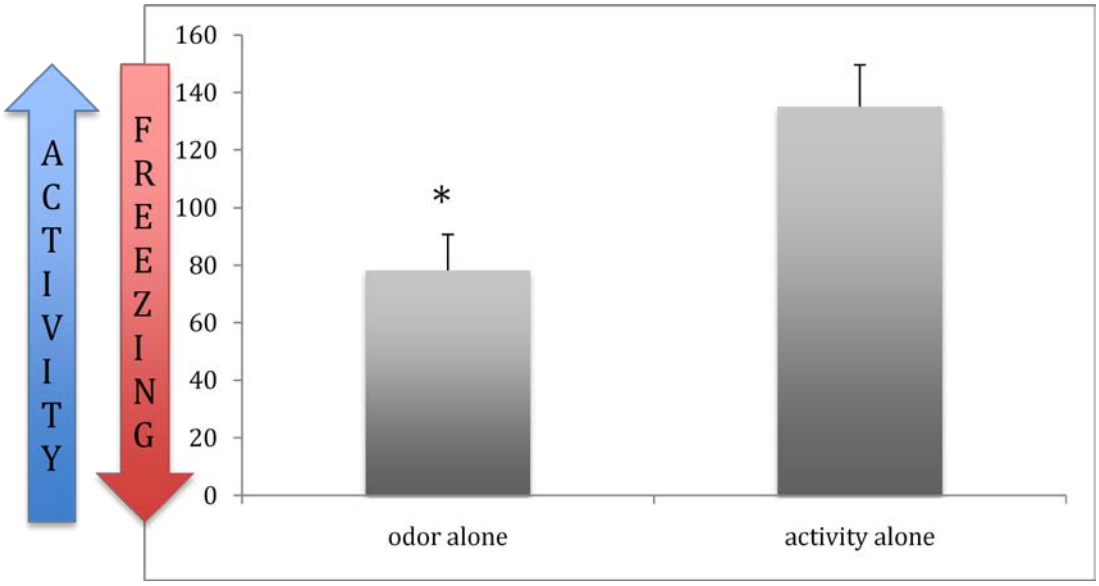

Figure 5. *Olfactory fear conditioning elicits freezing in mice*
Mice that were olfactory fear conditioned (n=23) exhibited a significant decrease in activity, or increase in freezing, when exposed to the odor CS alone, 24 hours following fear conditioning ($P<0.01$).

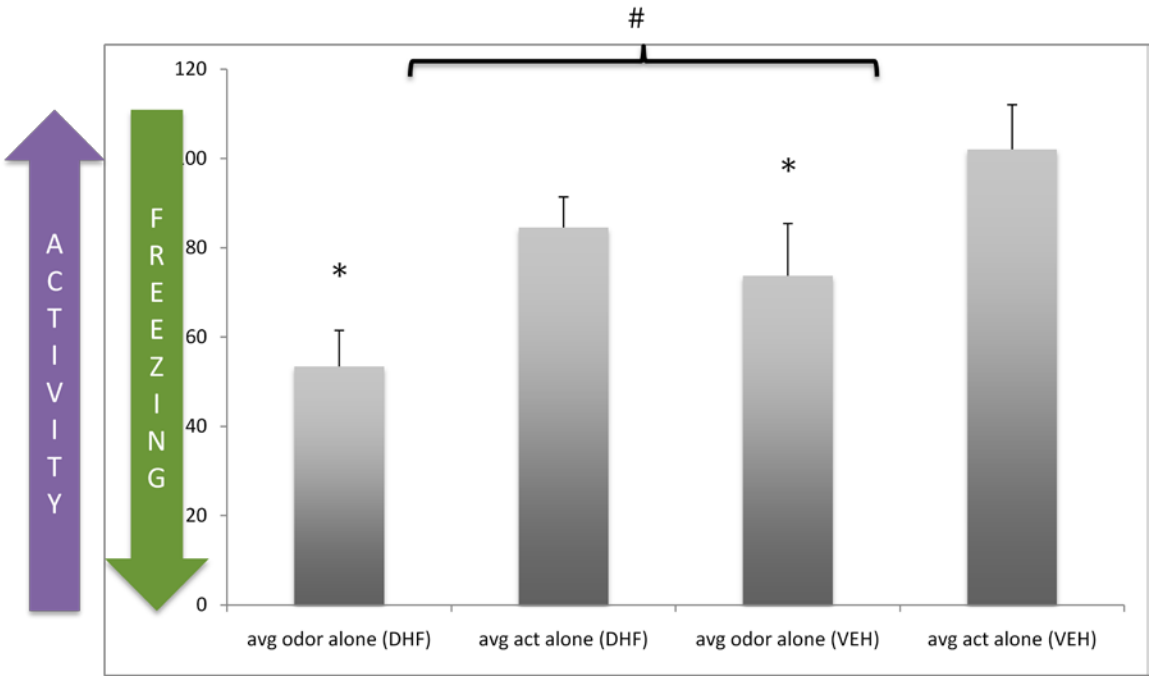

Figure 6. *Administration of 7,8-DHF prior to olfactory fear conditioning enhances learning.* Mice treated with 7,8-DHF (n=8) and vehicle (n=11) immediately before olfactory fear training showed significantly decreased activity when exposed to the odor alone 24-hours post fear conditioning ($p<0.05$). A main effect was shown for the drug, with mice that received 7,8-DHF showing an even greater decrease in activity than the vehicle group when exposed to the odor alone. Decreases in activity are equivalent to an increase in freezing.

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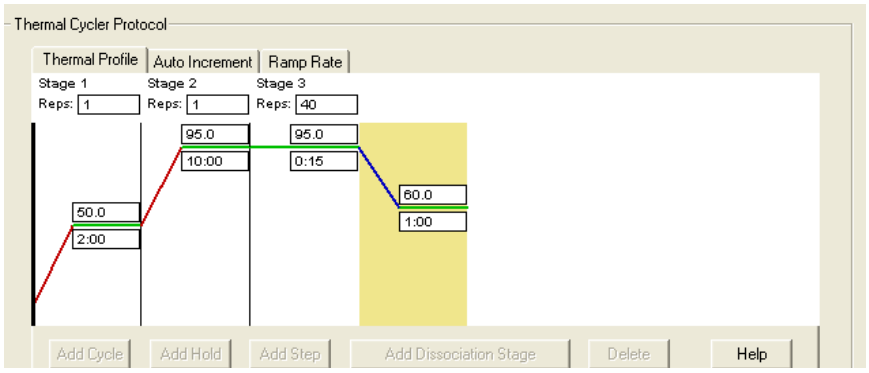

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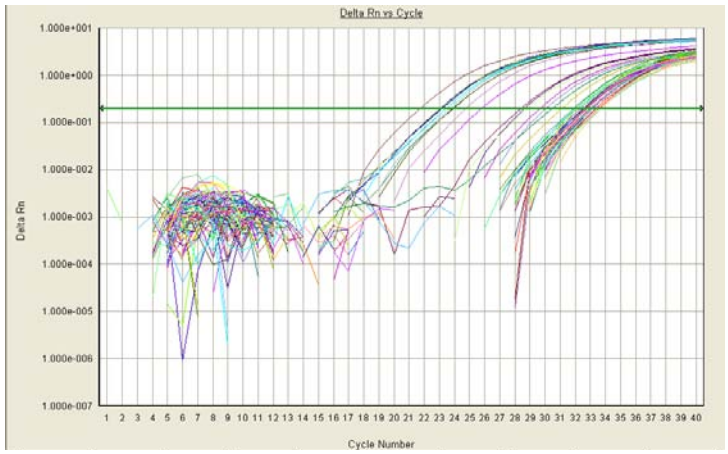

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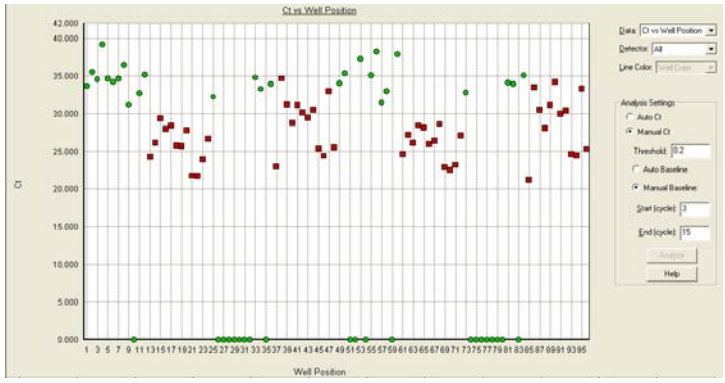

Figure 7. *Overview of rtPCR reaction*

The rtPCR reaction that was used to quantify BDNF mRNA completed 40 cycles of 15 second at 95 degrees C, during which cDNA strands are separated, followed by a 1 minute period at 60 degrees C, during which new strands of cDNA anneal (Figure 7a). The delta reaction vs. cycle graph shows the amplification of each sample of cDNA (Figure 7b). The cycle at which the amount of cDNA produced by the reaction reaches the threshold, designated by the green line, is termed the Ct value. The lower the cT value, the greater the quantity of cDNA. cDNA amplification levels off when the reactants are completely used up. Ct values of BDNF (green) and the control, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (red), for all samples are shown in Figure 7c. BDNF samples that did not have enough cDNA to reach the threshold during 40 cycles are shown to have Ct values of 0.

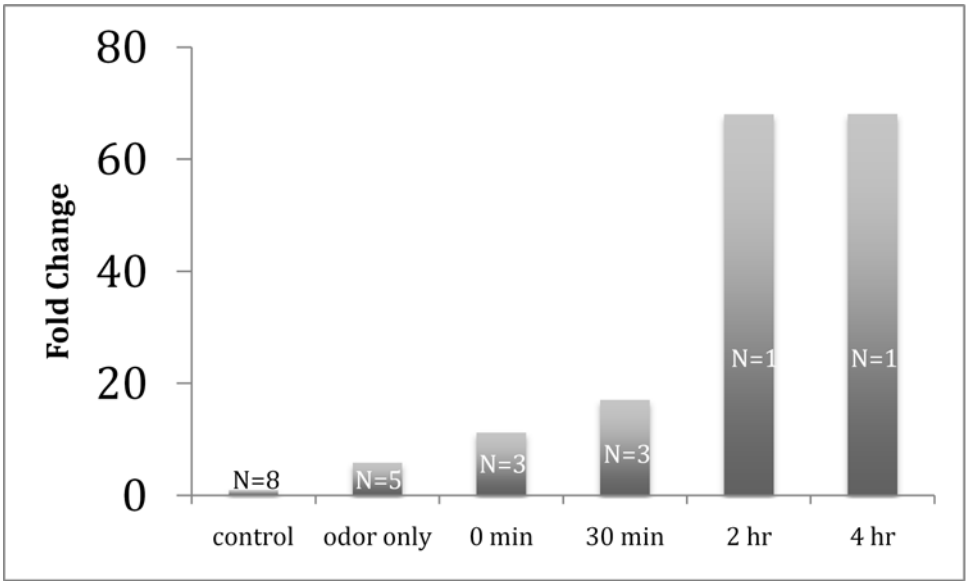

Figure 8. *BDNF mRNA increases at 2 and 4 hours post olfactory fear conditioning*
Fold change is a measure of the increase in cDNA over the course of the rtPCR reaction. BDNF mRNA from mice that received olfactory fear conditioning was shown to increase 2 and 4 hours after training. No increase in BDNF mRNA was shown in mice exposed to the odor alone or at 0 and 30 minutes post olfactory fear conditioning. This is preliminary data with some groups showing better success than others in reaching the threshold level after 40 rtPCR cycles. Those that did not reach threshold were eliminated from data analysis and prevented us from being able to complete statistical analyses.

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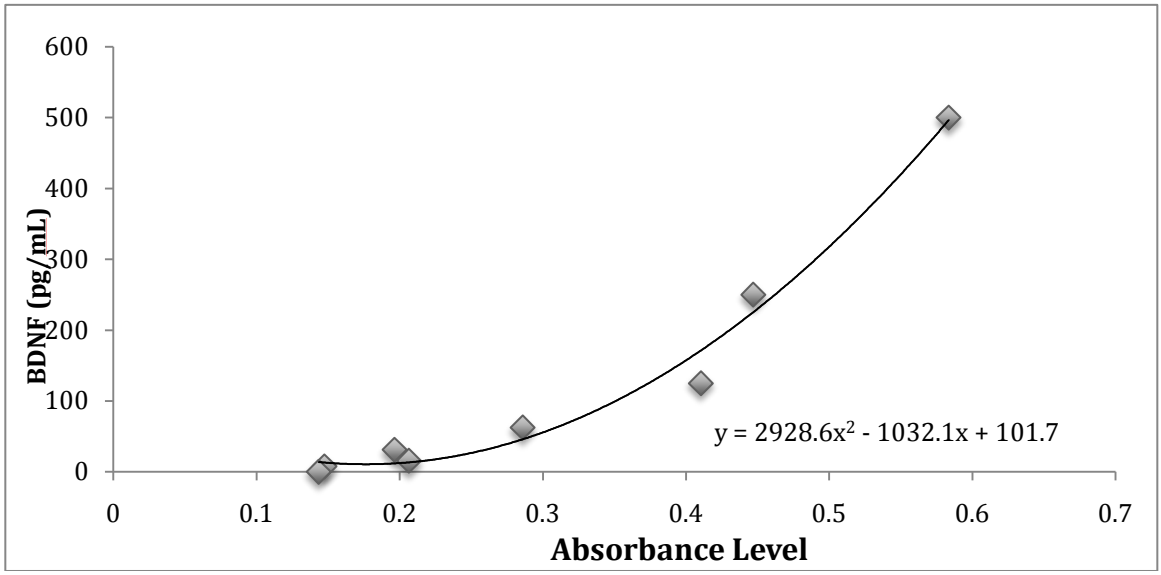

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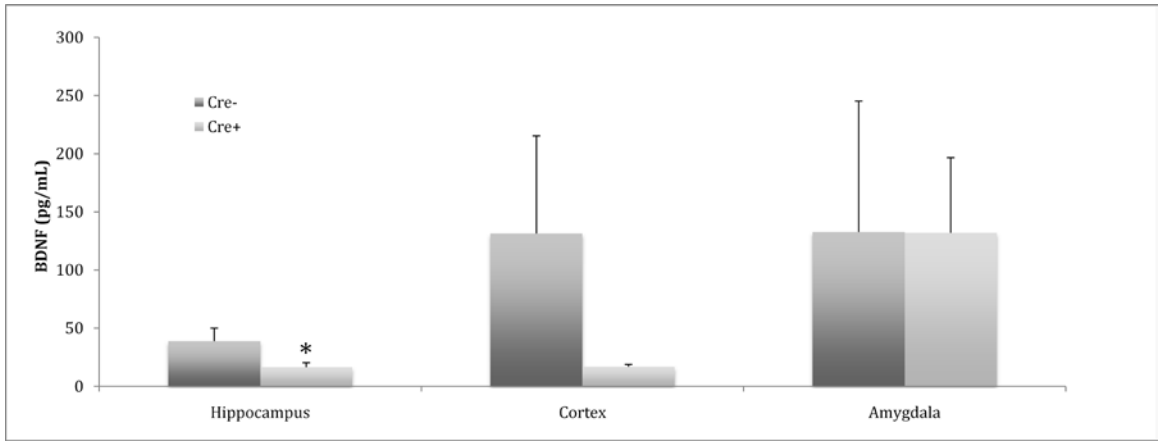

Figure 9. *BDNF protein expression in neocortical knockout mice in areas of the brain known express BDNF*

BDNF protein levels were looked at in BDNF knockout (Cre+) (n=12) and control mice (Cre-) (n=12) 30 minutes following tone/shock fear conditioning. BDNF protein levels were determined from a standard curve, which shows the relationship between the absorbance of the BDNF ELISA assay at 450nm and the concentration of BDNF (pg/ml) (Figure 9a). Neocortical BDNF knockout mice (Cre+) expressed significantly less BDNF protein in the hippocampus than mice expressing BDNF (Cre-) ($p<0.01$), but no differences were found in the prefrontal cortex and the amygdala (Figure 9b).

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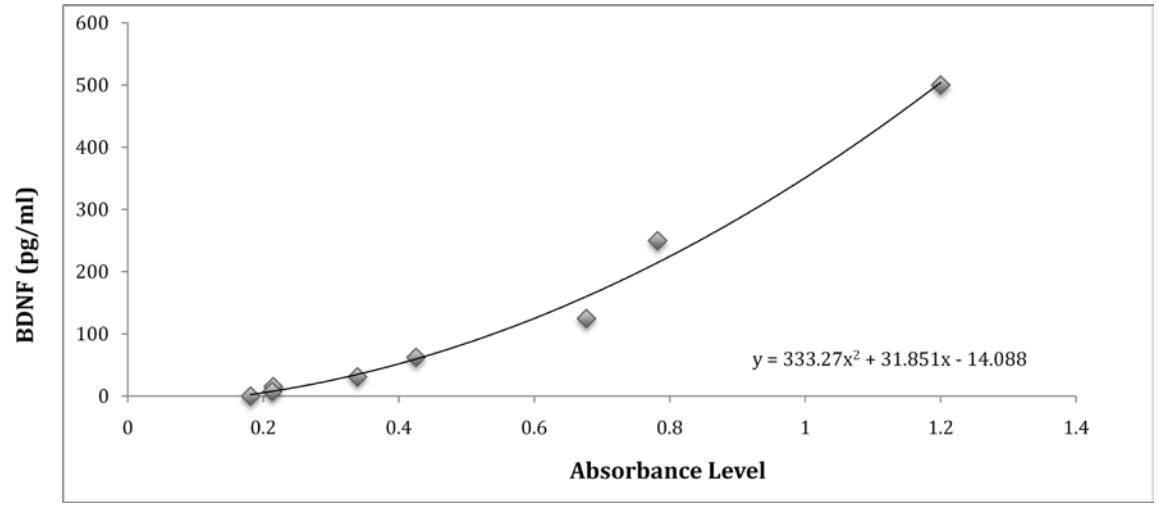

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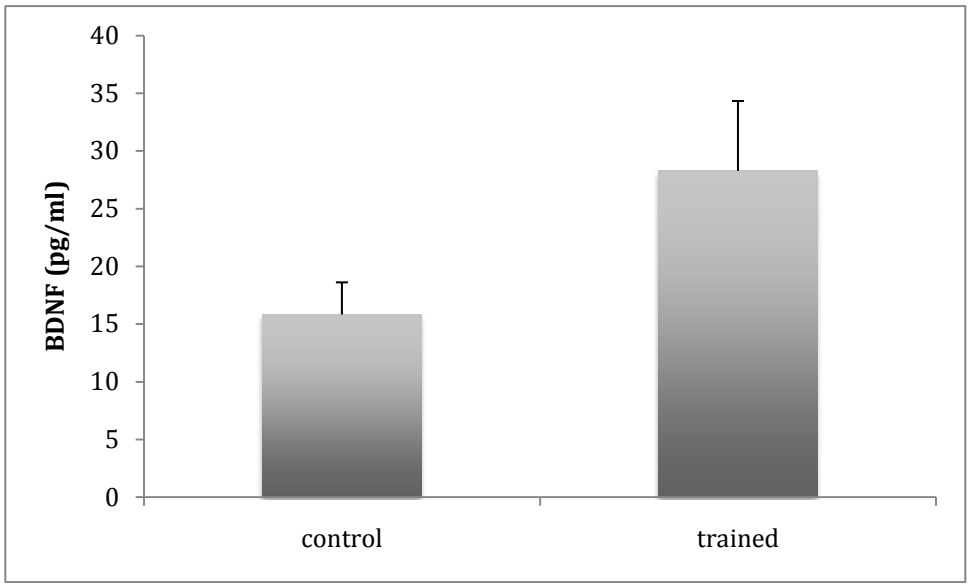

Figure 10. *BDNF levels in the olfactory bulb increase in mice that undergo olfactory-cued fear conditioning.*
Mice were olfactory fear conditioned (n=12) and their protein levels were examined using an ELISA assay. The standard curve shows the relationship between the absorbance of the BDNF ELISA assay at 450nm and the concentration of BDNF (pg/ml) (Figure 10a). Sample protein levels were determined from this curve. Mice that underwent olfactory-cued fear training showed increased levels of BDNF protein than the home-cage controls following olfactory fear conditioning ($p=0.05$).

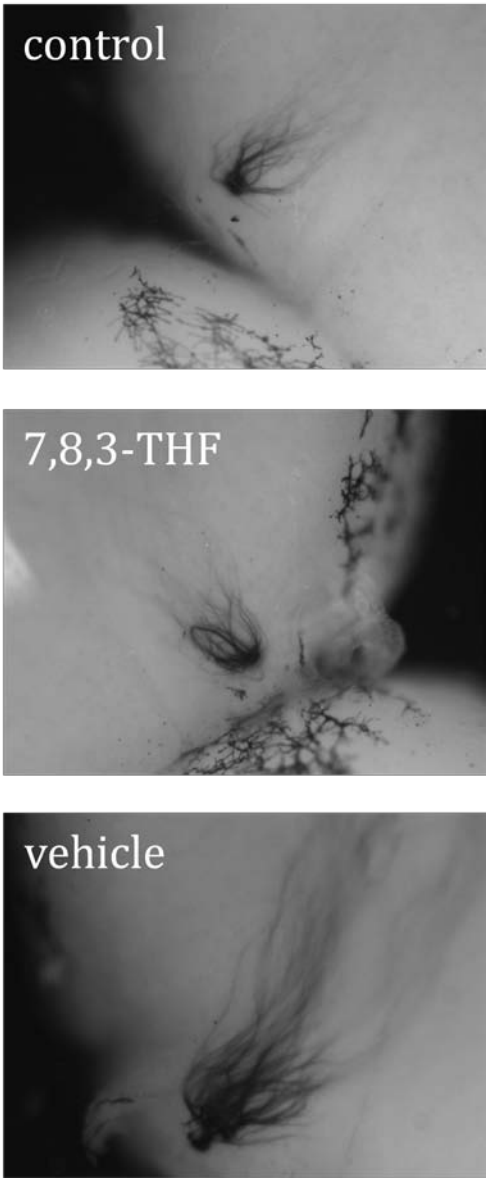

Figure 11. *M71 glomerular area increases following olfactory fear conditioning in vehicle treated mice*
Mice that were treated with vehicle (n=12) prior to olfactory fear conditioning showed an increase in M71 glomerular area as well as increased axon density, compared to control (n=12) and 7,8,3-THF (n=12) treated mice.

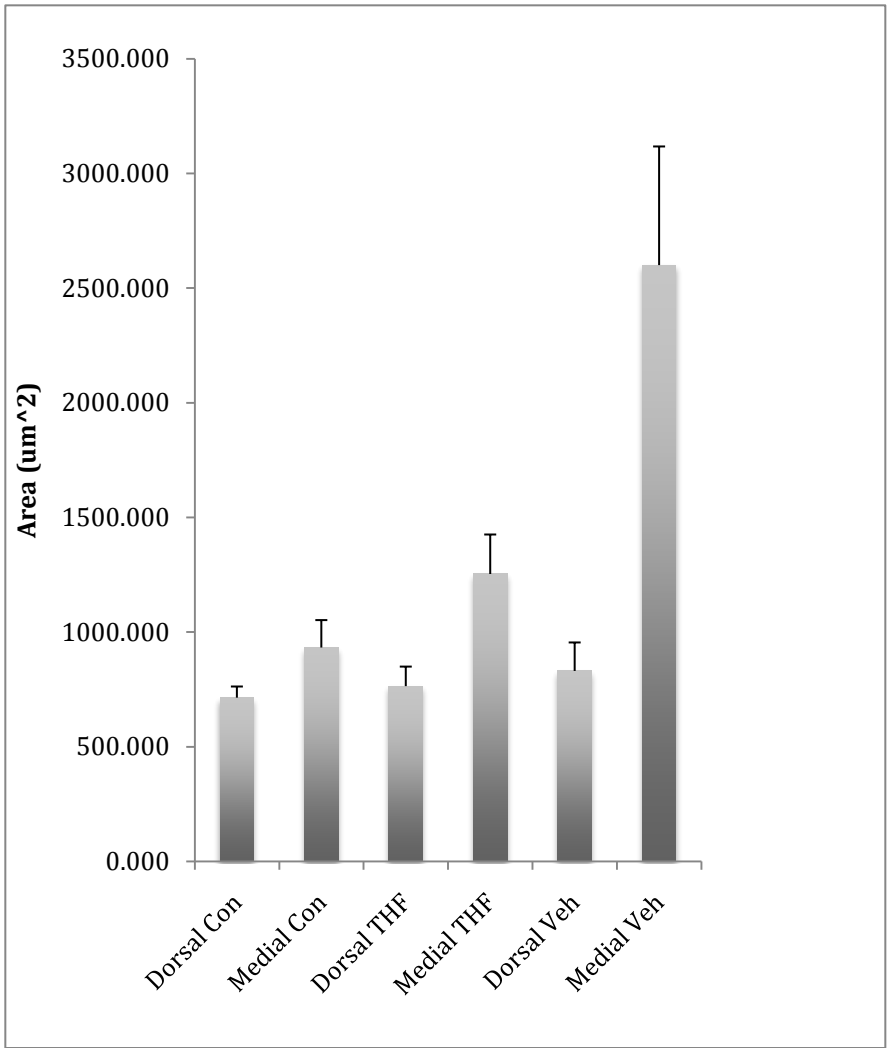

Figure 12. *The effects of administering TrkB agonists prior to olfactory-cued fear training on M71 glomerular area in M71–IRES–tauLacZ transgenic mice.*
Mice given vehicle or THF prior to olfactory-cued fear training did not show any significant differences in dorsal glomerular area; however, the medial glomeruli of the mice treated with vehicle had significantly greater area than the glomeruli of the mice treated with THF and the medial controls ($p<0.05$).

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