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TrkB Signaling: Beyond the Traditional Model

A dissertation submitted in partial satisfaction of the  
requirements for the degree Doctor of Philosophy  
in Neuroscience

by

Cortney Jill Crego

2013



ABSTRACT OF THE DISSERTATION

TrkB Signaling: Beyond the Traditional Model

by

Cortney Jill Crego

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2013

Professor Alcino Silva, Chair

In the decades since its discovery, our understanding brain-derived-neurotrophic-factor has undergone a transformation. Initially believed to only be expressed during development, BDNF has shown itself to be vital to a wide range of functions in the adult brain, including cell survival and differentiation, synaptic plasticity, and transcription. In turn, BDNF function has been implicated in a number of diseases including depression, cancer, schizophrenia, chronic pain, metabolic disorders, etc. As BDNF became a promising target of therapeutic interventions a more and more detailed assessment of its functioning and an understanding of its high affinity receptor, TrkB, emerged. BDNF-TrkB signaling has become one of the most studied and understood signaling pathways in the mammalian brain. As understanding led to dogma, research aims branched out to external influencers and consequences of this pathway and the functioning of this pathway has been subject to surprisingly little probing with modern, cutting-edge technologies, instead relying on data collected from studies that used the best possible

methods at the time, but now are showing their age. Here I will look beyond the conventional model at the implications of new methods and technologies that further reveal layers of intricacy in a signaling system already rife with complexity.

The dissertation of Cortney Jill Crego is approved.

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Abstracts:

**C.J. Crego**, Y.-S. Lee, A.J. Silva. (2010). "Molecular genetic studies of the role of TrkB signaling in hippocampal learning and memory." Society for Neuroscience, San Diego, CA.

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## Chapter 1 – Introduction

In the decades since its discovery, our understanding brain-derived-neurotrophic-factor has undergone a transformation. Initially believed to only be expressed during development, BDNF has shown itself to be vital to a wide range of functions in the adult brain, including cell survival and differentiation, synaptic plasticity, and transcription. In turn, BDNF function has been implicated in a number of diseases including depression, cancer, schizophrenia, chronic pain, metabolic disorders, etc. (Minichiello, 2009) As BDNF became a promising target of therapeutic interventions a more and more detailed assessment of its functioning and an understanding of its high affinity receptor, TrkB, emerged. BDNF-TrkB signaling has become one of the most studied and understood signaling pathways in the mammalian brain. As understanding led to dogma, research aims branched out to external influencers and consequences of this pathway and the functioning of this pathway has been subject to surprisingly little probing with modern, cutting-edge technologies, instead relying on data collected from studies that used the best possible methods at the time, but now are showing their age. Here we will look beyond the conventional model at the implications of new methods and technologies that further reveal layers of intricacy in a signaling system already rife with complexity.

BDNF-TrkB signaling has been studied and reviewed extensively (Minichiello, 2009; Cunha, 2010; Gupta, 2013; Panja, 2013; Zagrebelsky, 2013). The goal here is to look beyond the dogmatic model, but it will be reviewed briefly to give context to the growing evidence of its incompleteness. TrkB receptor activation by BDNF results in its dimerization and the autophosphorylation of tyrosine residues in its intracellular kinase domain. This

phosphorylation results in the recruitment of adaptor molecules, each with its own specific phosphotyrosine-binding domain. There are three main intracellular cascades that are activated by the TrkB receptor: the Ras-MAPK pathway, the PI3K-Akt pathway, and the PLC $\gamma$  pathway, each with its own distinct functionalities. The phosphorylation of the tyrosine at position 490 recruits Shc adaptor molecules, while phosphorylation of the tyrosine at position 816 activates the PLC $\gamma$  pathway (Cunha, et al., 2010). TrkB activates the PI3K pathway indirectly by way of other adaptor proteins (i.e. GRB-associated binder-q, insulin-receptor substrate 1 and 2) (Minichiello, 2009). A detailed schematic of the molecular pathways activated by TrkB can be seen in Figure 1.

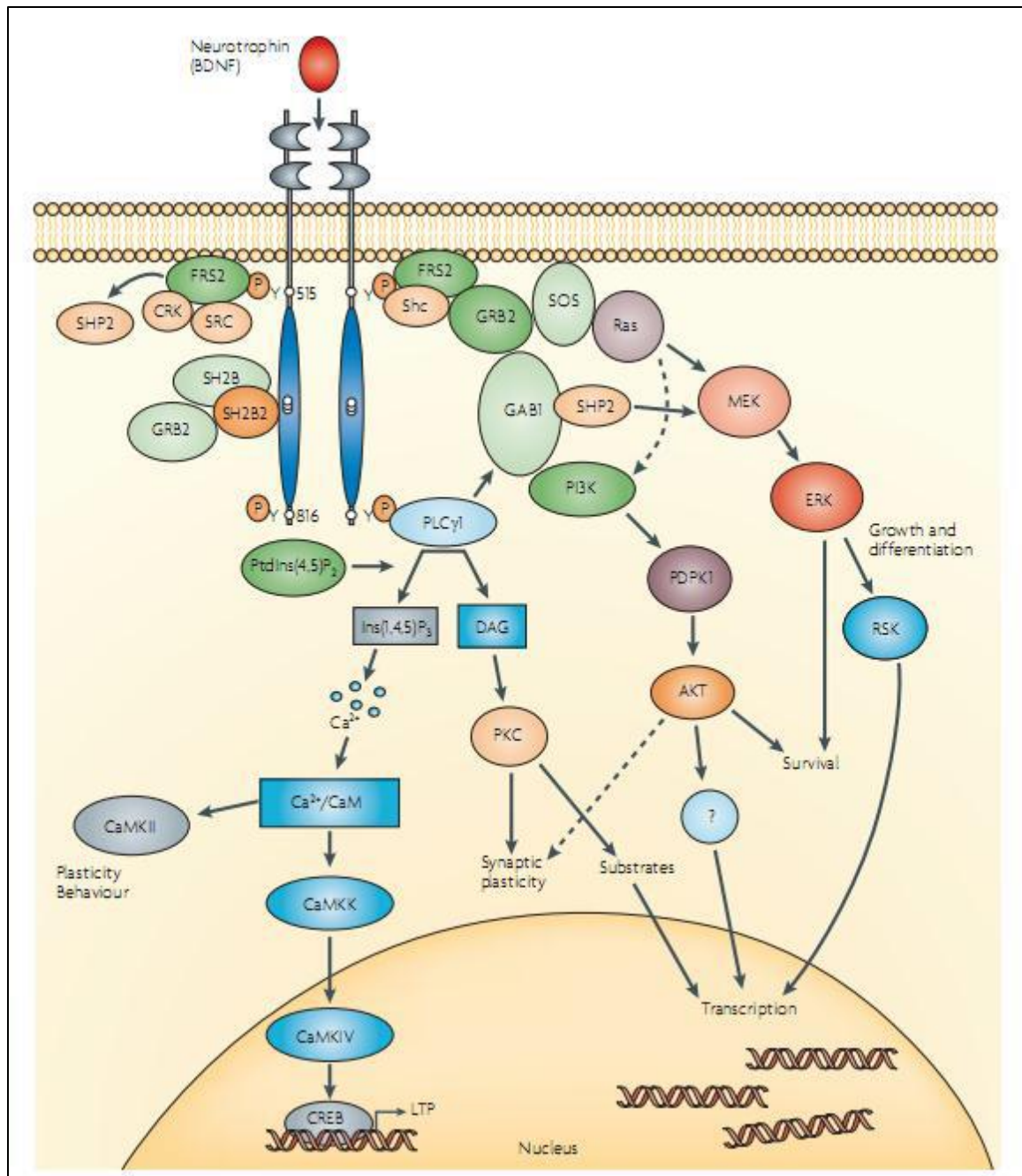


Figure 1. Schematic of TrkB signaling cascades (Minicheillo, 2009)

The recruitment and phosphorylation of Shc adaptors leads to the binding of growth factor receptor-bound protein 2 (GRB2) and/or son of sevenless (SOS) and the activation of the Ras-MAPK pathway (Huang, 2003). This pathway has been implicated

in proliferation, gene expression, differentiation, mitosis, cell survival, apoptosis, and plasticity (Minichiello, 2009; Gupta, 2013). Alternately, FRS2 also contains several phosphorylation-dependent recruitment sites for GRB2, which provides a Shc-independent mechanism for activation of Ras through the GRB2-SOS exchange factor complex. FRS2 also recruits numerous other signaling proteins, including the adaptor protein CRK, the tyrosine kinase SRC and a protein tyrosine phosphatase, SRC-homology phosphatase 2 (SHP2) (Kouhara, 1997; Wright, 1997; Hadari, 1998). Src sequentially recruits an intermediary protein Grb2 and the guanine nucleotide exchange factor SOS, initiates the GTP loading and activation of Ras and the activation of the Raf, MEK and ERK kinase cascade. Phosphorylated ERK translocates to the nucleus to activate transcription factors such as CREB to regulate gene expression. This complex response to receptor activation is believed to allow sustained activation of the MAPK pathway in response to neurotrophin binding (Reichardt, 2006).

As stated above, PI3K does not interact directly with Trk receptors. However, adaptor proteins such as GRB-associated binder-1 (GAB1), insulin-receptor substrate 1 (IRS1) and IRS2, which are recruited to activated TrkB via GRB2 binding, mediate the interaction and activation of PI3K by TrkB (Holgado-Madruga, 1997; Yamada, 1997). In response to this association, 3-phosphoinositides are generated by PI3K and activate 3-phosphoinositide-dependent protein kinase 1 (PDK1). Together with these 3-phosphoinositides, PDK1 activates the protein kinase Akt, which phosphorylates several proteins (Franke, 1997; Crowder, 1998). Phosphorylation of GAB proteins also leads to the formation of complexes that include SHP2 which enhances MAPK

signaling. BDNF was shown to facilitate local translation of proteins in dendrites by activation of mammalian target of rapamycin (mTOR) via PI3K. The PI3K pathway was shown to mediate the protective effects of BDNF in several neuronal cell types in vitro, including hippocampal neurons (Zheng, 2004).

Finally, phosphorylation of tyrosine 816 of TrkB leads to the recruitment of PLC $\gamma$ 1, which is then phosphorylated by the receptor (Kaplan, 2000). Activated PLC $\gamma$ 1 hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) to generate inositol-1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>) and diacylglycerol (DAG). Ins(1,4,5)P<sub>3</sub> promotes release of Ca<sup>2+</sup> from internal stores, which results in the activation of enzymes such as Ca<sup>2+</sup>/calmodulin-dependent protein kinases (Reichardt, 2006). The elevation of intracellular Ca<sup>2+</sup> is one of the most important biochemical outcomes of BDNF signaling in the postsynaptic cell. Evidence indicates that Ca<sup>2+</sup>-regulated mRNA translation occurs locally at postsynaptic sites. This mechanism provides a means for the rapid and accurate expression of activity-induced gene products such as BDNF at activated synapses. Moreover, the CaMKII activated transcription factor CREB has been shown to recognize CRE and a CaRE regulator elements in the *Bdnf* gene, activated its transcription. Thus, BDNF can regulate its own expression via activation of CaMKII signaling (Aakalu, 2001; West, 2002).

The differential activation and role of these cascades in neuronal survival will likely depend on the cell type and the involvement of specific physiological or pathological stimuli leading to regionally and temporally specific arrangements that will determine

outcome of specific activations of the receptor. The implication of this will be discussed at the end of the paper.

The role of multiple signaling pathways in learning and memory has been well documented, however the exact part that BDNF plays triggering and influencing these signaling pathways during learning remains to be discovered. For example, mutant mice where the PLC $\gamma$  phosphorylation site on TrkB has been disrupted show impaired hippocampal LTP and associative learning, demonstrating the importance of PLC $\gamma$  signaling in hippocampal plasticity mechanisms (Minichiello, 2002; Gartner, 2006; Gruart, 2007). In addition, crucial roles have already been shown for the ERK pathway, the PI3K-Akt pathway and the transcription factor CREB in synaptic plasticity and learning and memory (Sweatt, 2004; Thomas, 2004; Lin, 2001; Barco, 2003). As a result, BDNF can activate multiple signaling pathways that may act in a concerted fashion to regulate downstream cellular effects necessary for synaptic plasticity and memory formation. The interplay between these intracellular pathways needs to be further determined, but the degree to which each of them is activated is likely to depend on the levels of BDNF and TrkB, the temporal pattern of BDNF stimulation and whether the signaling is activated pre- or post-synaptically (Cunha, 2010).

The role of BDNF in learning and memory has been established by investigations in *in vivo* rodent models. BDNF mRNA expression increases in the hippocampus of rats following training in the Morris Water Maze (MWM), radial arm maze, passive avoidance, and contextual fear conditioning (Kesslak, 1998; Mizuno, 2000; Hall, 2000).

This indicates that the regulation of BDNF activity is a correlate of hippocampus learning in vivo. Interestingly, BDNF protein presents its highest expression in the hippocampus, neocortex, cerebellum, striatum and amygdala, all of which are responsible for cognitive functions (Kawamoto, 1996)). Its regulation by learning is also reflected in these brain regions. For example, increases in BDNF mRNA and protein levels and TrkB phosphorylation were found in the amygdala following fear conditioning (Rattiner, 2004a). Additionally, intra-hippocampal BDNF administration improved performance in MWM and pre-training infusions of anti-BDNF antibodies into the parietal cortex impaired inhibitory avoidance. Also, infusion of anti-BDNF antibodies into the parietal cortex impaired inhibitory avoidance by blocking CREB activation. These studies strongly suggest that BDNF has an essential role in the consolidation of LTM in a wide-range of behavioral protocols in wild-type animals.

Generally, the currently held consensus in the field is that increased levels of BDNF mRNA or protein results in an improvement in learning while a BDNF deficiency leads to a disruption in learning. While there is great support for this view of BDNF-TrkB signaling in the literature, new technologies and avenues of inquiry are continually revealing inconsistencies and outliers within this widely held dogma. Here I will explore mechanisms of action that exist outside the traditional model of BDNF-TrkB signaling.

## Chapter 2 – A New Model

The diversity of effects of BDNF/TrkB signaling in both molecular and temporal capacities presents an issue of confounding factors when imprecise methods are used. Until recently, studies were unable to account for the effects of long-term inactivation of BDNF/TrkB signaling. Genetic manipulations and chronic pharmacological inactivation of the pathway can have compounding effects on general brain development and function that go beyond the single task being tested. Even when acute inaction is achieved through the non-specific Trk antagonist, K252a, or the molecularly specific anti-BDNF antibodies or TrkB immunoglobulin G, the duration of action of these treatments may span the multiple waves of BDNF activity shown above and thereby have multiple effects that are not apparent in the final result. The mutant mouse strain developed by the Ginty lab (TrkB(f616a)) addresses this very issue (Chen, et al., 2005). Using a chemical-genetic approach, a mouse strain was created that is susceptible to molecularly specific and temporally precise inactivation of kinase function of its TrkB receptors. Through a point mutation that replaced a phenylalanine with an alanine at position 616 on the intracellular catalytic domain of the TrkB receptor (an ATP binding site), these receptors become sensitive to inactivation by a modified peptide kinase inhibitor (1NM-PP1). The inactivation by intraperitoneal (i.p.) injection of the peptides lasts just over 30 minutes, but in the absence of the peptide, the receptor is functionally normal (Chen, 2005). This precise control over the inactivation of TrkB makes this an ideal model for our studies.



In the following studies, several versions of the Morris Water Maze were used to test spatial learning. Variations were made in each protocol to allow for the specific time-course of each treatment. A detailed methods section can be found at the end of this chapter. The general structure of the water maze was as follows:

After 7-10 days of handling/habituation, mice were trained in the water maze with two blocks per day for 7 days. Each training block consisted of two back-to-back trials with 12 minutes between blocks. In each trial, mice were given 60 s to find the platform. Trials terminated when the mice found the platform. No single trial was allowed to go longer than 60 s. After each trial, mice were put on the platform for 15 s. On days 3, 5, and 7 the mice were given a 60 s probe test trial after the training trials, with the platform removed from the pool. The only protocol to use a different structure for the probe trials was the Injection Before and After Protocol. Because both an enhancement and a deficit were possible, probe trials were run on Days 2, 3, 4, 5, and 6 to allow greater sensitivity. To help avoid the extinction learning that may accompany daily probe trials, the duration of the probe trial was shortened to 30 seconds. An automated tracking system measured the average proximity, percent time spent in each quadrant, and the number of platform crossings in each quadrant. Table 1 gives schematic details of the protocol/injection variations. All 1NMPP1 injected doses were at a concentration of 16.6ng/g and were administered intraperitoneally. K252a was also delivered by i.p. injection at a dosage of 85mg/kg.

Protocol	Structure	Probe Trials on Days:
Injection Before Only		3, 5, 7
Injection After Only		3, 5, 7
Injection Before and After		2, 3, 4, 5, 6
K252a		3, 5, 7
Chronic		3, 5, 7

Table 1. Blue/numbered blocks represent training trials. Pink/P blocks represent probe/testing trails. All training trails ended when the submerged platform was located and did not persist longer than 60 seconds. All probe trials had a duration of 60 seconds, except in the Injection Before and After Protocol where probe trials were limited to 30 seconds.

As seen in Figure 2, only homozygous animals that received an injection of 1NMPP1 immediately prior to training showed selective searching during the Day 3 probe trial as measured by % Time by Quadrant (Hom/Drug - Paired t-test: T vs Adj:  $t=3.94(10)$   $p=.003$ , Paired t-test: T vs Opp:  $t=3.37(10)$   $p=.007$ ). They also show a significant difference in Average Proximity measures when compared to the other groups (One-way anova:  $F(3, 43) = 4.26$ ,  $p= .01$  Post-hoc – Fischer’s Least Significant Difference (LSD) – Het/Drug vs Hom/Drug  $p=.02$ , Het/Veh vs Hom/Drug  $p=.04$ , Hom/Drug vs Hom/Veh  $p=.04$ ). By Day 5 all groups show selective searching by % Time by Quadrant (Het/Drug: Paired t-test: T vs Adj: $t=6.59(15)$   $p=.001$ , Paired t-test: T vs Opp:  $t=3.54(15)$   $p=.003$ ; Het/Veh: Paired t-test: T vs Adj:  $t= 3.95(15)$   $p=.001$ , Paired t-test: T vs Opp: $t=3.84(15)$   $p=.002$ ; Hom/Drug: Paired t-test: T vs Adj:  $t= 3.63(9)$   $p=.006$ , Paired t-test: T vs Opp:  $t=3.08(9)$   $p=.01$ ; Hom/Veh: Paired t-test: T vs Adj:  $t=1.92(4)$   $p=.13$ , Paired t-test: T vs Opp:  $t=1.68(4)$   $p=.17$ ) and there are no significant difference between groups by Average Proximity.

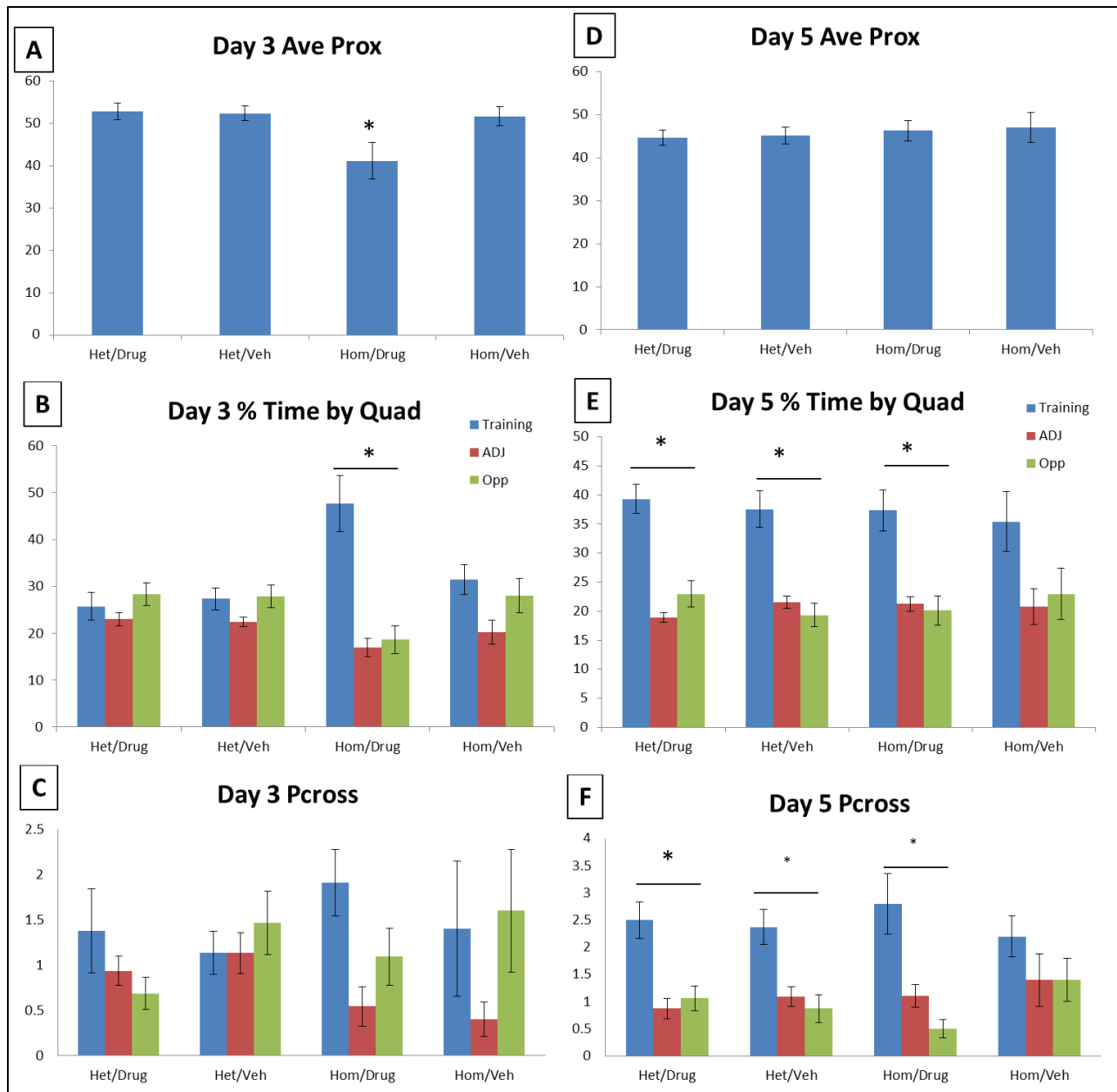


Figure 2 Homozygous animals that received treatment with 1NMPP1 prior to training show selective searching on Day 3. Het/Drug=16 Het/Veh=15 Hom/Drug=11 Hom/Veh=5. (A) There was a significant difference in Average Proximity measures when compared between groups (One-way anova:  $F(3, 43) = 4.26$ ,  $p = .01$  Post-hoc – Fischer’s Least Significant Difference (LSD) – Het/Drug vs Hom/Drug  $p = .02$ , Het/Veh vs Hom/Drug  $p = .04$ , Hom/Drug vs Hom/Veh  $p = .04$ ). (B) Only homozygous animals that received an injection of 1NMPP1 immediately prior to training showed selective searching during the Day 3 probe

trial as measured by % Time by Quadrant (Hom/Drug - Paired t-test: T vs Adj:  $t=3.94(10)$   $p=.003$ , Paired t-test: T vs Opp:  $t=3.37(10)$   $p=.007$ ). (E) By Day 5 all groups show selective searching by % Time by Quadrant (Het/Drug: Paired t-test: T vs Adj:  $t=6.59(15)$   $p=.001$ , Paired t-test: T vs Opp:  $t=3.54(15)$   $p=.003$ ; Het/Veh: Paired t-test: T vs Adj:  $t=3.95(15)$   $p=.001$ , Paired t-test: T vs Opp:  $t=3.84(15)$   $p=.002$ ; Hom/Drug: Paired t-test: T vs Adj:  $t=3.63(9)$   $p=.006$ , Paired t-test: T vs Opp:  $t=3.08(9)$   $p=.01$ ; Hom/Veh: Paired t-test: T vs Adj:  $t=1.92(4)$   $p=.13$ , Paired t-test: T vs Opp:  $t=1.68(4)$   $p=.17$ ) and (D) there are no significant difference between groups by Average Proximity.  $*=p<0.05$

This apparent enhancement in spatial learning was reflected in a 5-day contextual fear conditioning learning curve (Figure 3), where homozygous animals that received treatment with 1NMPP1 prior to training showed an increased freezing response.

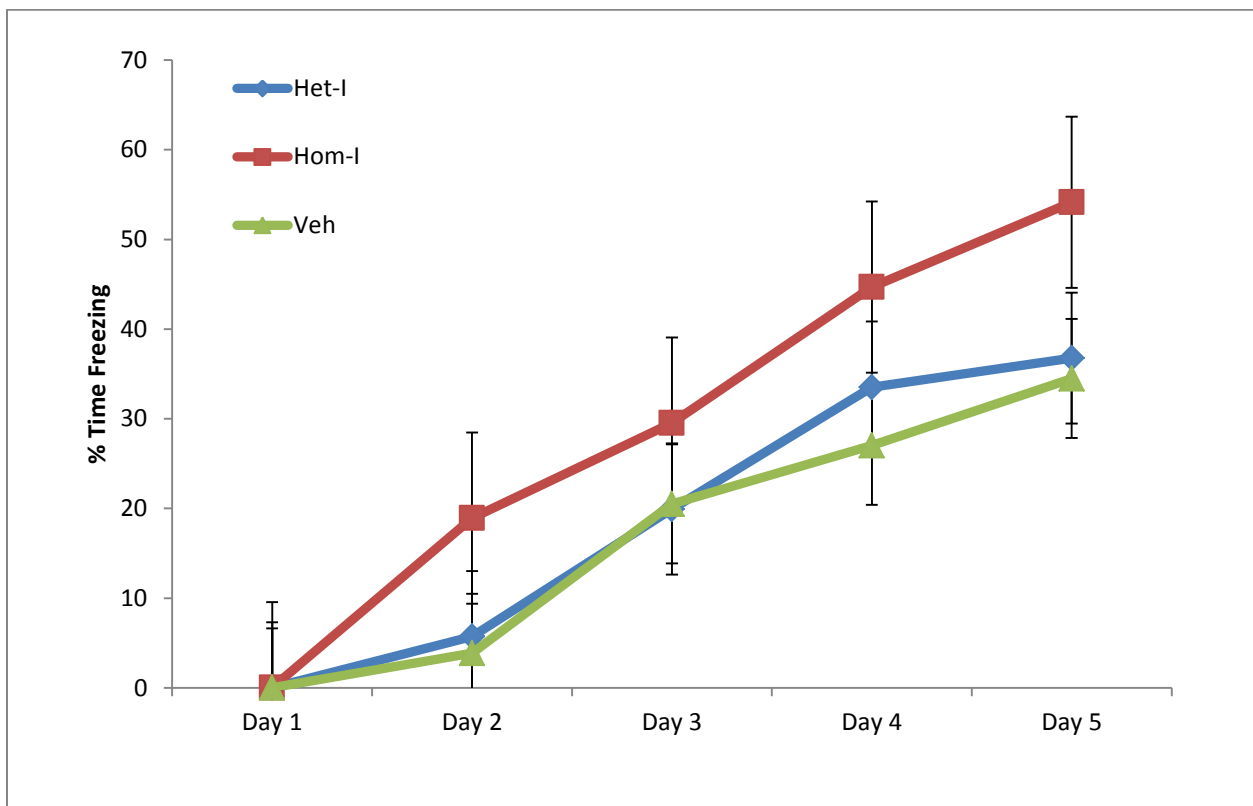


Figure 3. TrkB f616a homozygous mutants treated with 1NMPP1 show increased levels of freezing when compared with the other groups.

Due to the fact that heterozygous and homozygous animals in the vehicle injection condition perform nearly identically, they were combined into one group, and a one-way ANOVA was run on the regression slopes with three levels (heterozygous drug, homozygous drug, and vehicle). A post-hoc analysis consisting of the Dunnett *t*-test with correction for multiple comparisons tested both the heterozygous and homozygous drug conditions against the vehicle as a control condition, revealing a marginally significant difference between homozygous drug and vehicle,  $p = .057$ , and no significant difference between heterozygous drug and vehicle,  $p = .764$ .

Though approaching significance, the effect on treatment on learning during the contextual fear conditioning learning curve was dropped in favor of the more robust effect found during spatial learning during the Morris Water Maze for subsequent studies.

A protocol was developed and tested with the inactivation of TrkB taking place immediately after the last training trial. Confounding elements of the experimental design and the low number of animals used resulted in inconclusive results (not shown). Instead of continuing with this protocol, an alternative was developed with a balanced injection schedule allowing animals with TrkB inactivation either before or after training to be run simultaneously.

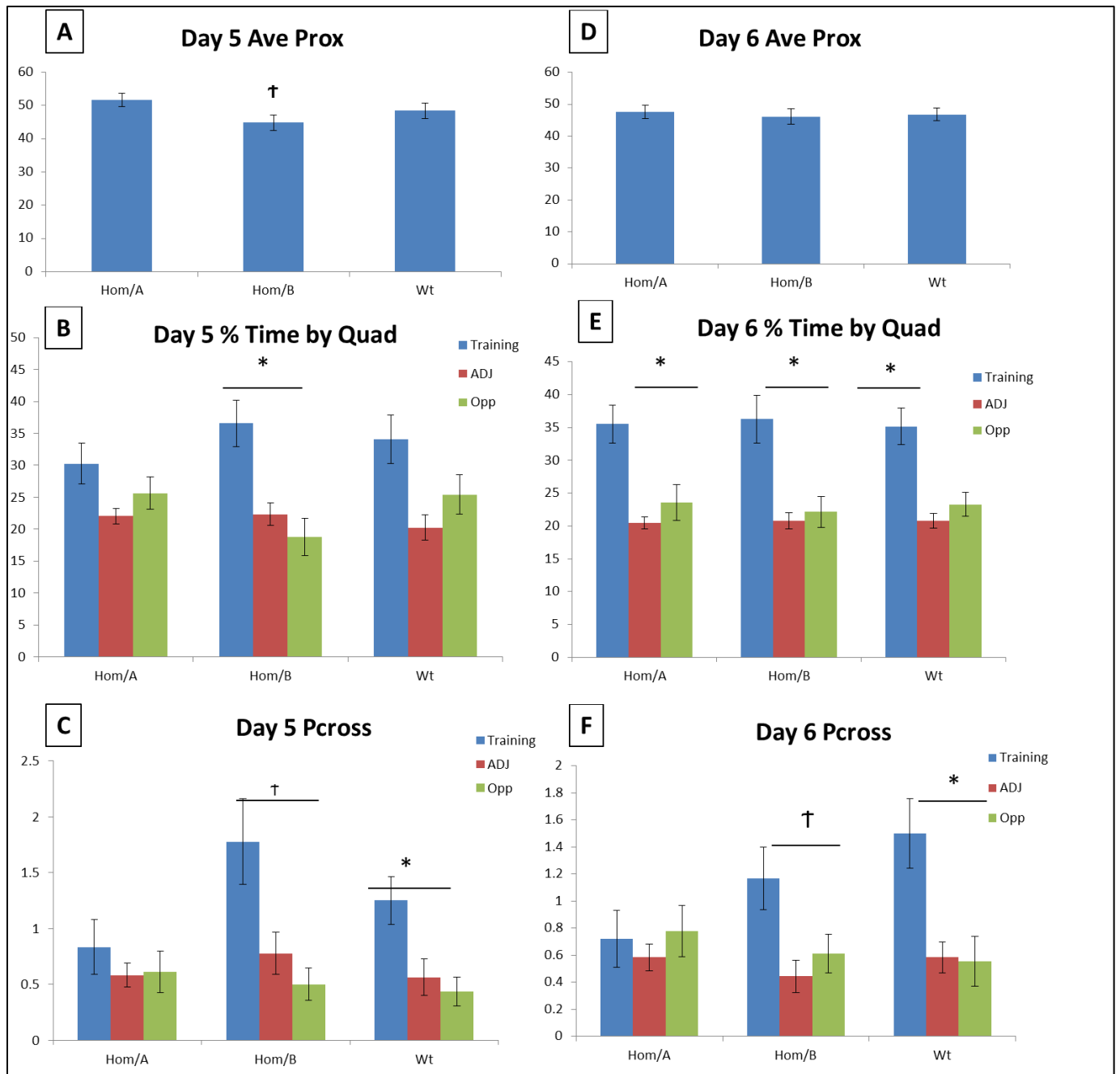


Figure 4 Homozygous animals that received treatment with 1NMPP1 prior to training were the first to show selective searching, followed by wildtype and homozygous animals that received treatment after training. Hom/B n=18, Hom/A=18 Wt=18 (A) There is a trend toward a significant difference between groups on the measure of Average Proximity (One-way anova:  $F(2,49) = 2.51$ ,  $p = 0.09$ ) and (B) only the group that received treatment with 1NMPP1 before training showed selective searching by % Day by

Quad on Day 5 (Hom/B: Paired t-test: T vs Adj:  $t=2.88(17)$   $p=.01$ , Paired t-test: T vs Opp:  $t= 3.20(17)$   $p=.005$ ). (C) Only the wildtype group shows selective searching by platform crossings on Day 5 (Wt: Paired t-test: T vs Adj:  $t= 2.18(15)$   $p=.05$ , Paired t-test: T vs Opp: $t= 3.57 (15)$   $p=.01$ ), though the homozygous animals that were treated with 1NMPP1 before training trended very nearly to significance (Hom/B: Paired t-test: T vs Adj:  $t=2.05(17)$   $p=.056$ , Paired t-test: T vs Opp:  $t=2.80(17)$   $p= .01$ ). By Day 6 of training, (E) all groups show selective searching as measured by % Time in Quadrants (Hom/A: Paired t-test: T vs Adj:  $t=4.40(17)$   $p=.001$ , Paired t-test: T vs Opp:  $t=2.26(17)$   $p=.04$ ; Hom/B: Paired t-test: T vs Adj:  $t=3.30 (17)$   $p=.004$ , Paired t-test: T vs Opp: $t=2.49(17)$   $p=.02$ ; Wt: Paired t-test: T vs Adj:  $t=2.83(17)$   $p=.001$ , Paired t-test: T vs Opp: $t=2.89(17)$   $p=.01$ ). (F) Only the wildtype group showed selective searching as measured by platform crossings (Wt: Paired t-test: T vs Adj:  $t=3.34(17)$   $p=.004$ , Paired t-test: T vs Opp:  $t=3.07(17)$   $p=.007$ ), though the homozygous animals that received treatment with 1NMPP1 before training trended very close toward significance on this measure (Hom/B: Paired t-test: T vs Adj:  $t=2.85(17)$   $p=.01$ , Paired t-test: T vs Opp: $t=2.0(17)$   $p=.07$ ).  $*=p<0.05$  and  $\dagger=p<0.10$ .

When injections were given both before and after training (either vehicle or 1NMPP1) (Figure 4), the first difference between treatment groups (and the first display of significant selective searching) is not seen until Day 5 of training and test trials. There is a trend toward a significant difference between groups on the measure of Average Proximity (One-way anova:  $F(2,49) =2.51$  ,  $p= 0.09$ ) and only the group that received treatment with 1NMPP1 before training showed selective searching by % Day by Quad on Day 5 (Hom/B: Paired t-test: T vs Adj:  $t=2.88(17)$   $p=.01$ , Paired t-test: T vs Opp:  $t= 3.20(17)$   $p=.005$ ). Only the wildtype group shows selective searching by platform crossings on Day 5 (Wt: Paired t-test: T vs Adj:  $t= 2.18(15)$   $p=.05$ , Paired t-test: T vs Opp: $t= 3.57 (15)$   $p=.01$ ), though the homozygous animals that were treated with 1NMPP1 before training trended very nearly to significance (Hom/B: Paired t-test: T vs



Adj:  $t=2.05(17)$   $p=.056$ , Paired t-test: T vs Opp:  $t=2.80(17)$   $p=.01$ ). By Day 6 of training, all groups show selective searching as measured by % Time in Quadrants (Hom/A: Paired t-test: T vs Adj:  $t=4.40(17)$   $p=.001$ , Paired t-test: T vs Opp:  $t=2.26(17)$   $p=.04$ ; Hom/B: Paired t-test: T vs Adj:  $t=3.30(17)$   $p=.004$ , Paired t-test: T vs Opp:  $t=2.49(17)$   $p=.02$ ; Wt: Paired t-test: T vs Adj:  $t=2.83(17)$   $p=.001$ , Paired t-test: T vs Opp:  $t=2.89(17)$   $p=.01$ ). Only the wildtype group showed selective searching as measured by platform crossings (Wt: Paired t-test: T vs Adj:  $t=3.34(17)$   $p=.004$ , Paired t-test: T vs Opp:  $t=3.07(17)$   $p=.007$ ), though the homozygous animals that received treatment with 1NMPP1 before training trended very close toward significance on this measure (Hom/B: Paired t-test: T vs Adj:  $t=2.85(17)$   $p=.01$ , Paired t-test: T vs Opp:  $t=2.0(17)$   $p=.07$ ).

Though efforts were made to reduce extinction learning due to the daily probe trials, it is possible that the less robust learning displayed by the Hom/B group may be due to enhanced extinction. It would be interesting in the future to test this form of learning directly. Despite any possibly complications due to extinction, selective searching was first seen by animals that received inactivation prior to training, followed by wildtype animals, then animals that were inactivated after training, though this final group showed only a subtle deficit. A more robust learning deficiency (mimicking expectations from literature) may be achievable with a longer duration of inactivation.

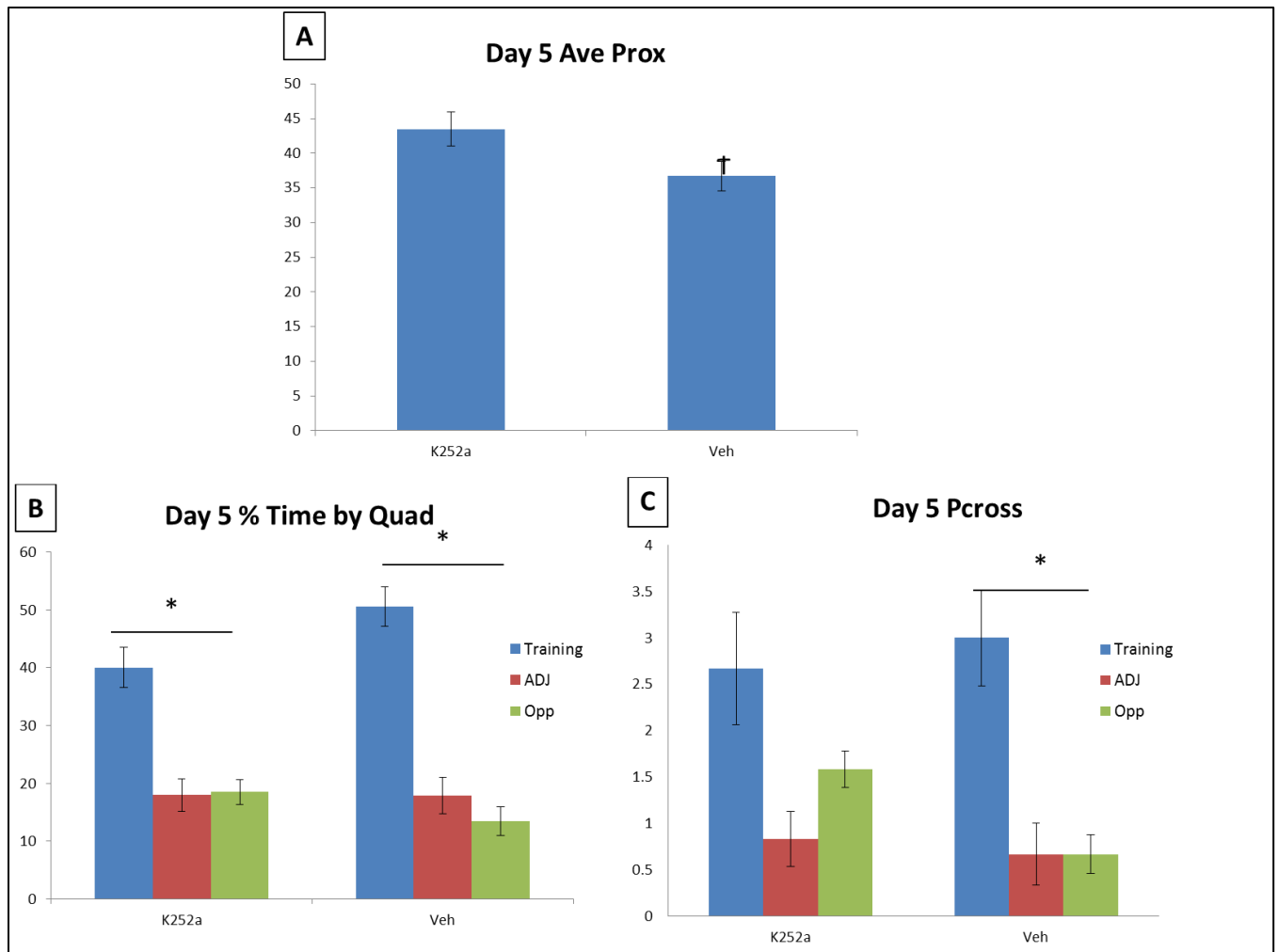


Figure 5. Wildtype animals treated with K252a before training show a slight deficit in spatial learning. K252a =12 Veh = 6 (A) There was a trend towards significantly better average proximity in wildtype animals that received treatment with the vehicle (One-way anova:  $F(1,16) = 3.15$ ,  $p = .095$ ). (B) Both groups showed selective searching as measured by % Time in Quadrant (K252a: Paired t-test: T vs Adj:  $t=4.50(11)$   $p=.001$ , Paired t-test: T vs Opp:  $t= 3.94(11)$   $p=.002$ ; Veh: Paired t-test: T vs Adj:  $t= 7.23(5)$   $p=.001$ , Paired t-test: T vs Opp:  $t=7.12(5)$   $p=.001$ ), (C) but only the vehicle treated group show selective searching as measured by platform crossings (Veh: Paired t-test: T vs Adj:  $t= 4.38(5)$   $p=.007$ , Paired t-test: T vs Opp:  $t=4.18(5)$   $p= .009$ ). \*= $p<0.05$  and †= $p<0.10$ .

To test the standard protocol developed with the use of 1NMPP1 with a commonly used non-selective Trk inhibitor, the original protocol was run with inactivation being induced by K252a. As seen in Figure 5, selective searching was first seen on Day 5 test trials. There was a trend towards significantly better average proximity in wildtype animals that received treatment with the vehicle (One-way anova:  $F(1,16) = 3.15$ ,  $p = .095$ ). Both groups showed selective searching as measured by % Time in Quadrant (K252a: Paired t-test: T vs Adj:  $t=4.50(11)$   $p=.001$ , Paired t-test: T vs Opp:  $t= 3.94(11)$   $p=.002$ ; Veh: Paired t-test: T vs Adj:  $t= 7.23(5)$   $p=.001$ , Paired t-test: T vs Opp:  $t=7.12(5)$   $p=.001$ ), but only the vehicle treated group show selective searching as measured by platform crossings (Veh: Paired t-test: T vs Adj:  $t= 4.38 (5)$   $p=.007$ , Paired t-test: T vs Opp:  $t=4.18(5)$   $p= .009$ ).

It was surprising a more robust effect was not seen, but a number of factors may have been to blame. K252a is widely acknowledged as a non-specific inhibitor of all the Trk family kinases as well as some downstream targets, and so opposing effects may be been working at odds. Typically, this particular inhibitor is used to prove the BDNF-dependency of another treatment/effect, but not used on its own, making the ideal dosage difficult to discern and so a more ideal result may be have been achievable with a higher dose. Further exploration of this issue was not pursued for two main reasons. First, the field in general would not benefit as newer, more refined methods of inactivation are increasingly becoming available (such as the mutant mouse used in these studies) rendering further development of K252a activity moot. Second, this

project in particular would not benefit as there is evidence to suggest that K252a is unable to bind to the mutated TrkB receptor of these mice.

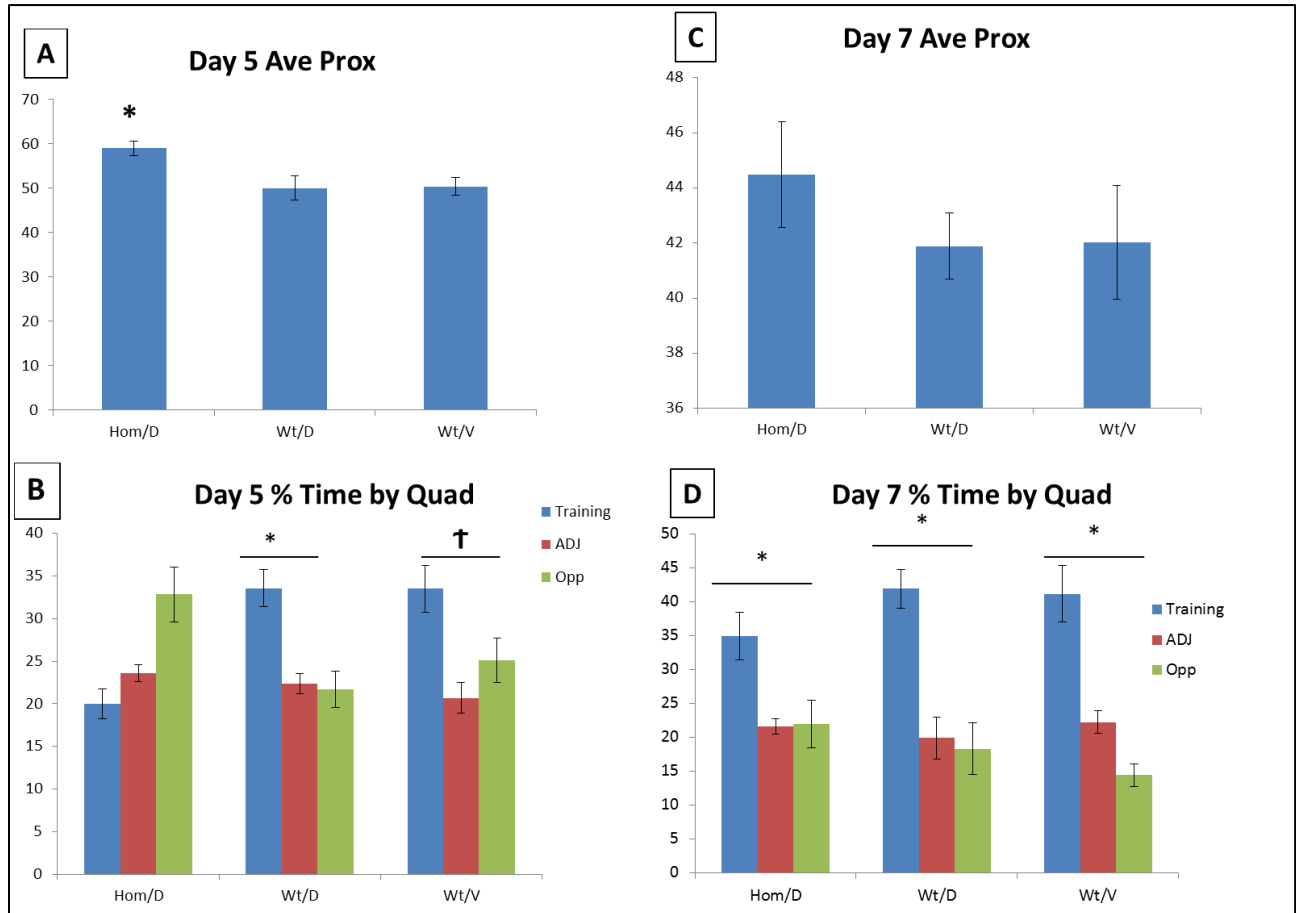


Figure 6 Chronic inactivation of TrkB via drinking water results in a deficiency in spatial learning.

Hom/D=10 Wt/D = 8 Wt/V= 7 By Day 5 of training, (A) there was significantly worse average proximity for Hom/D (One-way anova:  $F(2,22) = 6.14$ ,  $p = .008$ ; Post-hoc – Fischer’s Least Significant Difference (LSD) – Hom/D vs Wt/D  $p = .006$ , Hom /D vs Wt/D  $p = .009$ ).

(B) Also only Wt/D show significant searching, but Wt/V are trending as measured by % Time In Quadrant (Wt/D: Paired t-test: T vs Adj:  $t = 3.81(7)$   $p = .007$ , Paired t-test: T vs Opp:  $t = 3.41(7)$   $p = .01$ ; Wt/V: Paired t-test: T vs Adj:  $t = 3.04(6)$   $p = .02$ , Paired t-test: T vs Opp:  $t = 2.11(6)$   $p = .08$ ).

(C) By Day 7 all show selective searching by % Time by Quadrant (Hom/D: Paired t-test: T vs Adj:  $t = 3.29(9)$   $p = .009$ , Paired t-test: T vs Opp:  $t = 2.00(9)$   $p = .08$ ; Wt/D:

Paired t-test: T vs Adj:  $t=3.80(7)$   $p=.007$ , Paired t-test: T vs Opp:  $t=9.27(7)$   $p=.001$ ; Wt/V: Paired t-test: T vs Adj:  $t=3.30(6)$   $p=.02$ , Paired t-test: T vs Opp:  $t=4.90(6)$   $p=.003$ . \* $p<0.05$  and † $p<0.10$ .

Finally, to replicate the deficiencies seen in other studies with this mutant (Lu, 2011; Choi, 2012) where TrkB was inactivated chronically, 1NMPP1 treatment was administered via drinking water and run through the original watermaze protocol without injections. Homozygous animals that were received 1NMPP1 in their drinking water showed a deficiency in spatial learning in the Morris Water Maze (Figure 6). By Day 5 of training, there was significantly worse average proximity for Hom/D (One-way anova:  $F(2,22) = 6.14$ ,  $p=.008$ ; Post-hoc – Fischer's Least Significant Difference (LSD) – Hom/D vs Wt/D  $p=.006$ , Hom /D vs Wt/D  $p=.009$ ). Also only Wt/D show significant searching, but Wt/V are trending as measured by % Time In Quadrant (Wt/D: Paired t-test: T vs Adj:  $t=3.81(7)$   $p=.007$ , Paired t-test: T vs Opp:  $t=3.41(7)$   $p=.01$ ; Wt/V: Paired t-test: T vs Adj:  $t=3.04(6)$   $p=.02$ , Paired t-test: T vs Opp:  $t=2.11(6)$   $p=.08$ ). By Day 7 all show selective searching by % Time by Quadrant (Hom/D: Paired t-test: T vs Adj:  $t=3.29(9)$   $p=.009$ , Paired t-test: T vs Opp:  $t=2.00(9)$   $p=.08$ ; Wt/D: Paired t-test: T vs Adj:  $t=3.80(7)$   $p=.007$ , Paired t-test: T vs Opp:  $t=9.27(7)$   $p=.001$ ; Wt/V: Paired t-test: T vs Adj:  $t=3.30(6)$   $p=.02$ , Paired t-test: T vs Opp:  $t=4.90(6)$   $p=.003$ ).

Taken together these studies give strong evidence that timing is a crucial element when discerning the role of TrkB signaling in spatial learning. Unfortunately, attempts at an independent replication of this data have, so far, been unsuccessful. As seen in Figure 7, no enhancement in spatial learning was seen in the homozygous animals that received treatment with 1NMPP1 immediately prior to training. Preliminary statistics

reveal, however, that wildtype animals that received injections of 1NMPP1 immediate prior also do not show selective searching for the target quadrant (Wt/D: Paired t-test: T vs AdjR:  $p=0.01$ , Paired t-test: T vs AdjL:  $p=0.03$ , Paired t-test: T vs Opp:  $p=0.10$ ).

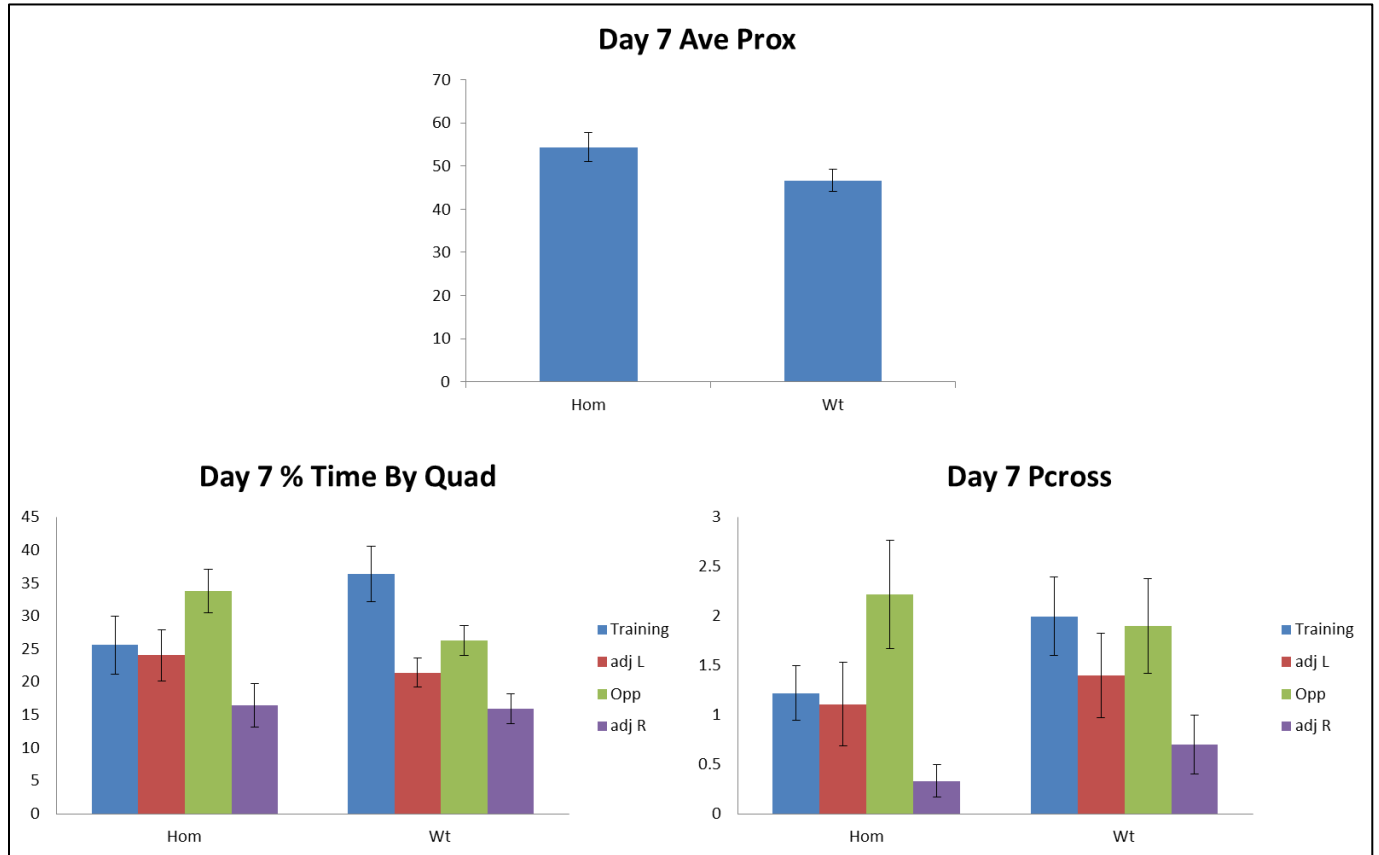


Figure 7 Wildtype animals that received injections of 1NMPP1 immediate prior also do not show selective searching for the target quadrant Hom = 10, Wt = 10. (Wt/D: Paired t-test: T vs AdjR:  $p=0.01$ , Paired t-test: T vs AdjL:  $p=0.03$ , Paired t-test: T vs Opp:  $p=0.10$ ).

## Methods

### *Transgenic mice.*

Generation of *TrkB(f616a)* mice has been described (Chen, 2005) and were generously provided by the Stryker Lab. They were maintained on a JacBl6 background for several generations to generate a common genetic background. The animal protocols used in the studies here were approved by the UCLA Institutional Animal Research Committee.

### *Morris water maze behavioral test*

To habituate animals to investigator contact and procedural elements of subsequent water maze training, mice were handled for 7-days prior to training on the Morris watermaze (for approximately 2 min/animal/day). This included repeatedly picking up the animals by the tail and holding them on the hand. On the last 3 days of handling, animals were habituated to several scruff restraints that would be used to administer i.p. injections during the training. Subsequently, mice were trained on the hidden version of the water maze; the escape platform was hidden underneath the water surface in a constant location of the pool. The water in the pool (diameter: 1.2 m) was made opaque with white non-toxic paint (water temperature: 22–24C). Animal behavior was monitored with an automated system (HVS water). Animals were entered into the pool from one of six randomly assigned starting positions. Trials were completed when the animal climbed on the escape platform or when 60 s had elapsed, whichever came first. Animals remained on the escape platform for 15 s after completion of trials. We gave

four daily training trials for 5-7 consecutive days. Each training block consisted of two back-to-back trials with 12 minutes between blocks. The exact timing of injections, training, and testing varied by paradigm and is explained in detail in Table 1. To assess how accurately animals had learned the position of the escape platform, we gave a probe trial after completion of training on specific days. During the probe trial, the escape platform was removed from the pool and we determined the proportion of time that the animal spent searching in the target quadrant (which previously contained the escape platform) or the other quadrants. Probe trial data were analyzed in two ways. To determine if animals spent more time in the target quadrant than the other quadrants (which is indicative of preferential searching in the target quadrant), we compared target quadrant occupancy to the average occupancy of the other quadrants by paired t-test. Average Proximity measures were compared between groups using One-way ANOVA and when significance was found a Post-hoc test (Fischer's Least Significant Difference) was run.

### *Contextual fear conditioning*

Training consisted of placing the mice in a conditioning chamber and presenting a shock (2 s, 0.65 mA) 40 seconds later. Mice were removed immediately after the shock. Mice received training in the same box for 5 consecutive days. Our index of memory, freezing (the cessation of all movement except for respiration), was assessed by an automated scoring system (Med Associates) during the first 30 seconds of each training trial with a 18 frames per second sampling; freezing was counted if the mouse froze



continuously for 1 second or more. Differences between genotype/treatment groups were measured with One-way ANOVA. A post-hoc analysis consisting of the Dunnett *t*-test with correction for multiple comparisons tested both the heterozygous and homozygous drug conditions against the vehicle as a control condition.

### *Drug*

1NMPP1 (Cayman Bio) was administered by i.p. injection at a concentration of 16.6ng/g or delivered via drinking water at a concentration of 125mM. In both delivery systems 1NMPP1 was first dissolved in 100% DMSO and then diluted to less than 0.02% DMSO in phosphate buffered saline solution (PBS). K252a (Sigma) was also delivered by i.p. injection at a dosage of 85mg/kg, dissolved in DMSO and diluted to less than 0.02% DMSO in PBS.

### Chapter 3 - Beyond the Conventional Model

There is evidence that there are some TrkB signaling cascades that are not dependent on kinase function and so are free to exert their influence even in the presence of inhibitors, such as K252a, or BDNF scavengers, such as TrkB-IgG. Huang and McNamara found transactivation of TrkB by zinc, which results in phosphorylation of tyrosine 505/506, is not inhibited by the blockade of BDNF binding or by blocking ATP binding by K252a or 1NM-PP1 (Huang, 2009). However, zinc-mediated transactivation at tyrosine 515 is kinase dependent as it was blocked by K252a and 1NM-PP1.

Trk activation is associated with activation of a variety of G-protein coupled receptors (GPCRs) including A2a adenosine receptors, PAC1 receptors for the neuropeptide pituitary adenylate cyclase activation peptide (PACAP), and endocannabinoid receptors. GPCR-dependent Trk activation differs from neurotrophin-dependent Trk activation in one important aspect. Neurotrophins necessarily activate Trk receptors residing in the plasma membrane, whereas GPCR signaling causes activation of Trk proteins that may initiate before they exit the Golgi apparatus and this activation may actually impede the trafficking of Trk proteins to the plasma membrane (Rajagopal, 2004; Schecterson, 2010a). GPCR-mediated Trk transactivation in neurons is dependent on activation of Fyn. Although the mechanism by which these GPCRs activate Fyn in neurons experiencing Trk transactivation has not been characterized, it is known that GPCR-dependent release of the beta-gamma subunits from G proteins activates Src Family Kinases. A multitude of GPCRs are expressed in the nervous system, an important

question is whether only a few GPCRs have the capacity to transactivate Trks, and, if so, what determines this.

Transactivation of Trks, however, is not limited to GPCRs alone. Trk transactivation is induced by agonist-dependent activation of Low-density lipoprotein receptor-related protein 1 (LRP1), and by zinc ion, which is co-released during glutamatergic neurotransmission (Huang, 2008; Shi, 2009). In both cases, the effect is mediated by Fyn activation. The time-dependence of Trk transactivation varies dramatically in these various systems, and corresponds with differences in the time-dependence of Fyn activation in each system. For example, zinc ion activation of Fyn and TrkB takes place within seconds, whereas adenosine agonists and PACAP require several hours to achieve maximum activation of Fyn and TrkB (Huang, 2008). Evidence concerning the range of physiological functions of Trk transactivation is limited, but, there is strong evidence for such function. For example, while TrkB activation in the mossy fiber pathway of hippocampus has been shown to play a critical role in kindling in the model of epileptogenesis in mice, mouse strains conditionally lacking BDNF display relatively normal kindling. Apparently this is because TrkB activation during kindling is mainly mediated by zinc ion release during neurotransmission (Huang, 2008).

A recent study (Vries, et al., 2009) further highlights the fact that our understanding of TrkB signaling is still evolving, in sometimes surprising ways. The current model of TrkB signaling calls for Shc adaptor molecules to be recruited after activation of TrkB by BDNF. Using Bioluminescent Resonance Energy Transfer (BRET) assays, Vries, et al.

instead found evidence that Shc can exist as a preformed complex with TrkB that has constitutive activity, demonstrating another possibility for kinase-independent activity and further contributing to the complexity of TrkB signaling cascades.

TrkB is activated through the process of dimerization in response to BDNF binding. This reaction is subject to the thermodynamic law of mass action, meaning that high local membrane density of Trk proteins may promote spontaneous activation in the absence of neurotrophins (Schechter, 2010b). Although such spontaneous activation has been observed for many receptor tyrosine kinases under scenarios of overexpression, the Trk proteins are particularly prone to this behavior (Schechter, 2010b). Although this apparent hair-trigger event of Trk activation has been a continual annoyance for investigators studying Trk signaling mechanisms, it may be argued that leaving Trk proteins maintained at the brink of activation represents an essential feature of Trk function, as it allows diverse forms of modulation of the cellular environment to impinge on Trk activation.

The idea that BDNF/TrkB signaling affects learning and memory in a time-dependent manner is supported by several previous studies (Alonso et al., 2000, Bekinschtein, et al., 2008). Alonso used a single-trial learning task and inactivated BDNF signaling at various time-points before and after training and then tested both short-term and long-term memory. Animals received step-down avoidance training along with infusions of anti-BDNF antibodies at 15 minutes prior, immediately after, or 1 hour after training. When tested 1.5 hours after training, only the animals infused 15 minutes prior to

training showed a deficit in their short-term memory. In a test of long-term memory, the animals received an infusion 15 minutes prior, immediately after, 1 hour, 4 hours, or 6 hours after training and were tested 24 hours later. When tested, those animals that received an anti-BDNF infusion at 15 minutes prior, 1 hour, and 4 hours after training all showed a reduced latency to step-down compared to those that received infusions immediately or 6 hours after training, suggesting a second wave of BDNF was necessary to establish the long-term memory. Demonstrating an even later wave of BDNF synthesis that is crucial to long-term memory, Bekinschtein, et al. showed that blocking protein synthesis 12 hours after inhibitory avoidance training impaired memory when tested 7 days after training, but not 1 day after. This was shown to be BDNF-dependent when co-administration of human recombinant BDNF with anisomycin 12 hours after training rescued the memory deficit at day 7. This infusion of hrBDNF at 12 hours after training was sufficient to establish retention of the learned fear at 7 days after training, even when a weak training protocol that normally would not induced a long-lasting memory was used (Bekinschtein, et al., 2008). The necessity of multiple waves of BDNF signaling to establish long-term memory emphasizes the need for precise temporal control over inactivation of BDNF/TrkB signaling. Such temporal control is possible through the use of our mutant mouse model.

In addition to its activation of multiple molecular cascades and its time-specific action, BDNF is able to act both pre- and post-synaptically on both excitatory and inhibitory neurons (Cunha, et al., 2010). Historically, more attention has been paid to BDNF/TrkB's modulation of excitatory circuits, but recent evidence suggests that its

influence on GABA release and inhibitory circuitry plays a substantial role in learning and memory (Vaz, et al., 2006). Evidence from our lab suggests a possible convergence of action between TrkB activation of the MAPK/ERK pathways and neurofibromin regulation of Ras. Nf1 is a GTPase activated protein (GAP) that acts to transform Ras from its active to its inactive state. When Nf1 action is altered, as is the case in Neurofibromatosis type 1, Ras remains in its active state longer, resulting in greater stimulation of the MAPK/ERK pathway. Cui, et al. found that Nf1 regulation of ERK signaling, by way of Ras, increases GABA release and contributes to subsequent learning deficits (Cui et al., 2008). This idea is supported by evidence that a reduction in MAPK activity decreases synapsin 1 phosphorylation and GABA release (Jovanovic, et al., 2000). It has already been shown that TrkB activates the MAPK/ERK pathway by way of its Shc binding site (Minicheillo, 2009) and so it seems logical that TrkB inactivation may reduce GABA release by modulation of ERK signaling and act to rescue the phenotype of the Nf1 mouse model.

## Chapter 4 – Conclusion and Future Directions

Studies in this lab and in others have shown that the modified peptide 1NMPP1 does not have an effect on wildtype animals (Chen, 2005, Sallert, 2009; Liu, 2013). Other studies in this lab have shown that wildtype B16 mice that receive 4 training trials per day can typically be expected to show selective searching for the target quadrant in less than 7 days of training, and so it is unusual that they failed to do so in this experiment. While it is discouraging not to get independent confirmation, I do not believe this nullifies the previous findings, but rather highlights the limitations of using a multi-trial, multi-day learning task that cannot be attributed to a well-defined learning circuit in conjunction, with such a specific form of inactivation. While these first experiments do well to demonstrate the need for further investigation, they are not the ideal method of inquiry. Suggestions for further study are described below.

It is possible that this mutant model is allowing access to a newly isolated time-frame and may be exposing an inequity in response to TrkB activation during training that was previously hidden by inexact methods of inactivation. In addition, if the activation of molecular cascades downstream of TrkB is dependent on the levels of available adaptor proteins, it stands to reason that different subsets of neurons, with different levels of available adaptor molecules, will react differently to the activation (and therefore, inactivation) of TrkB. As we become more and more precise in our manipulations of signaling cascades we are likely to find evidence that the intricate balance of molecular

reactions after TrkB activation is highly dependent on timing, cell type, and the method of manipulation.

Previous studies have shown the regional and temporal specificity of TrkB signaling (Panja, 2013). Knowing now the unreliability of these behavior tasks, I would suggest a turn to the molecular scale studies. It is possible that given the interconnectedness of learning behavior at a systemic level, that any results may be washed out by difference responses in different regions and other compensatory mechanisms. Preliminary results from Western Blot analysis for each of the major TrkB downstream pathways were inconclusive, but these studies were done with whole Hippocampal samples and some electrophysiology studies have shown differential activation of the molecular pathways between LTP induced by high frequency stimulation vs theta burst stimulation and CA3 vs CA1 (Panja, 2013). While this mouse model does give access to molecularly specific inactivation of TrkB, it is expressed throughout the brain and so any inactivation on the systemic level loses out on regional specificity. I would suggest the next study look at in situ immunohistochemistry or other in vitro methods that allow discernment of regional specificity of activation of downstream cascades and to let these findings dictate further behavioral confirmations in conjunction with the use of other mutant mouse models (i.e. Shc and PLC $\gamma$  phosphorylation site mutants).

If the observed enhancements from the first studies withstand further scrutiny or can be replicated with newly developed protocols I believe a simple mechanism will be found to



underlie this surprising results. Again and again in this paper there have been demonstrations of just how perpetually active the TrkB receptor is. In addition to its role as the high affinity receptor for BDNF, which already comes with proposed multiple waves of activity in both the initiation and maintenance of new and old memories, it is also subject to transactivation via multiple sources, its own basal level of activity, and spontaneous dimerization with matching full length monomers and truncated isoforms. Always on the precipice of activation, it is constantly engaged in some activity. I propose that the enhancement seen here was the result of a temporary relief and reset of the TrkB receptor. Its short-term inactivation stripped away competing mechanisms, and once again allowed to participate in the absence of its selective inhibitor 1NMPP1, a significantly increased proportion of TrkB receptors were at the ready to respond to the multiple waves of BDNF triggered by the training trials performed as part of the water maze. If true, this mechanism of reset and optimization could be used to identify which pathways are triggered in response to particular learning tasks and help clarify the tangled web of BDNF-TrkB signaling. A depiction of this proposed mechanism can be seen in Figure 8.

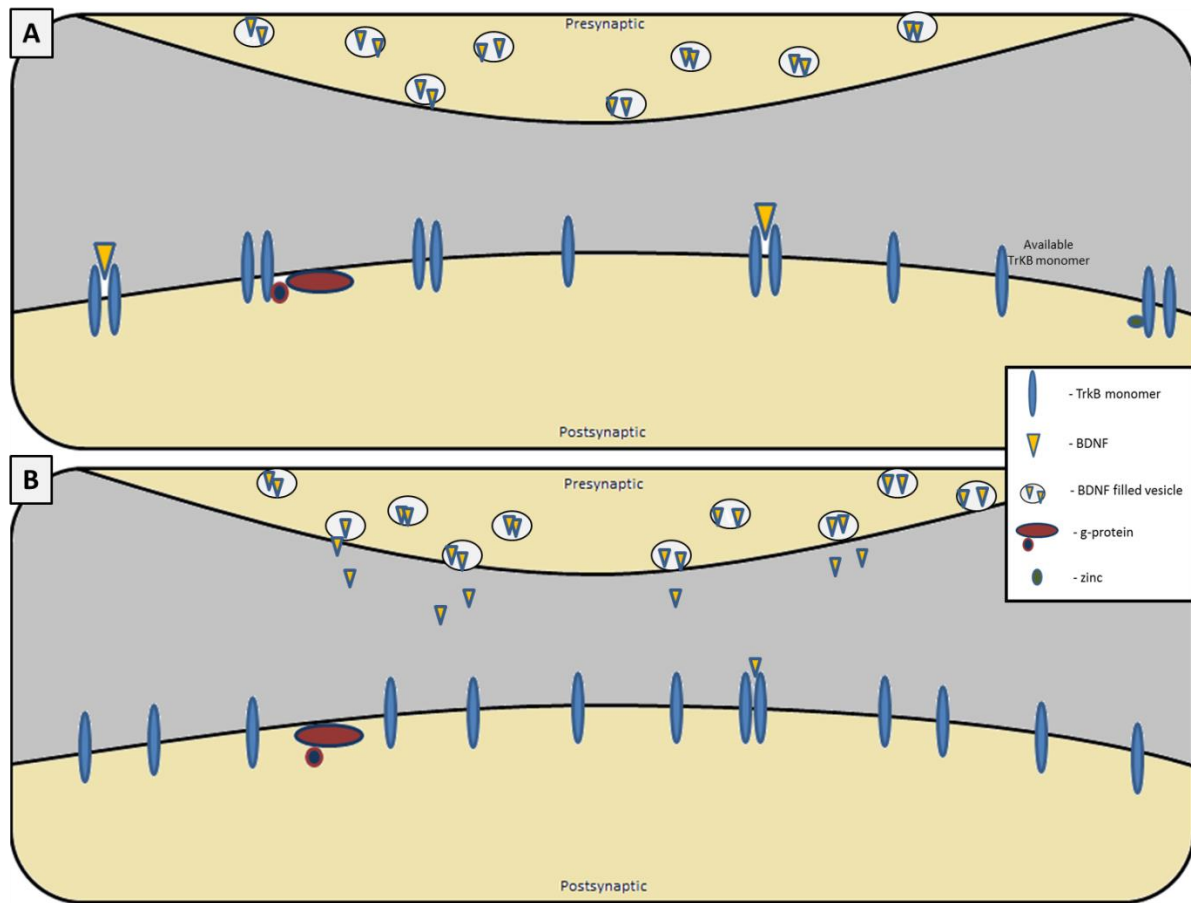


Figure 8. A schematic representation of the proposed mechanism underlying the learning enhancements described in this paper. (A) Pre- and post-synaptic cells prior to inactivation by 1NMPP1. TrkB monomers exist in multiple states, a certain percentage of which are in their active state, driven by their high-affinity ligand, BDNF, transactivated by either zinc or g-proteins, or spontaneously active homodimers. (B) Pre- and Post-synaptic cells after inactivation by 1NMPP1. With some or all driven and spontaneous activity halted by the administration of 1NMPP1, a greater percentage of TrkB monomers are available for dimerization and activation via the driving force of the learning event (presumably excretion of BDNF), thus allowing greater concerted activity in correlation with the learning event.

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