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Role of TrkB in the Anxiolytic-like and Antidepressant-like Effects of Vagal Nerve Stimulation: Comparison with Desipramine

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Abstract

A current hypothesis regarding the mechanism of antidepressant action suggests the involvement of brain derived neurotrophic factor (BDNF). Consistent with this hypothesis, the receptor for BDNF (and neurotrophin 4/5), Tropomyosin related kinase B (TrkB), is activated in rodents by treatment with classical antidepressant drugs. Vagal nerve stimulation (VNS), a therapy for treatment resistant depression, also activates TrkB in rodents. However, the role of this receptor in the therapeutic effects of VNS is unclear. In the current study, the involvement of TrkB in the effects of VNS was investigated in rats by using its inhibitor, K252a. Anxiolytic-like and antidepressant-like effects were analyzed using the novelty suppressed feeding test and forced swim test, respectively. K252a blocked the anxiolytic-like effect of chronic VNS treatment and the antidepressant-like effect of acute VNS treatment. By contrast, blocking TrkB did not prevent either the anxiolytic-like or antidepressant-like effect of chronic treatment with desipramine, a selective noradrenergic reuptake inhibitor; it did, however, block the acute effect of desipramine in the forced swim test. To examine whether the activation of TrkB caused by either VNS or desipramine is ligand-dependent, use was made of TrkB-Fc, a molecular scavenger for ligands of TrkB. Intraventricular administration of TrkB-Fc blocked the acute activation of TrkB induced by either treatment, indicating that treatment-induced activation of this receptor is ligand-dependent. The behavioral results highlight differences in the involvement of TrkB in the chronic effects of an

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Author Contributions

Conceived and designed experiments: AS, AF and FC. Performed experiments or assisted during experiments: AS, FC, HW and YC. Analyzed the data: AS, FC and AF. Wrote the paper: AS, AF.

Conflict of Interests

Dr. A. Frazer has served on advisory boards for Lundbeck, Takeda Pharmaceuticals International, Inc. and Eli Lilly and Co. In the past, Dr. Frazer has received financial compensation as a consultant for Cyberonics Inc. and has also obtained grant support from them for a preclinical study. None of the other authors have any conflicting interests to declare.

antidepressant drug and a stimulation therapy as well as its role in acute versus chronic effects of desipramine.

Keywords

Antidepressants; Vagal Nerve Stimulation; TrkB; BDNF; Desipramine; Depression

1. Introduction

Most antidepressants (ADs) enhance transmission in serotonergic and/or noradrenergic systems (Lenox and Frazer, 2002, Morilak and Frazer, 2004) forming the basis of the monoaminergic theory of depression (Prange, 1964, Schildkraut, 1965, Gyermek, 1966). The neurogenesis theory posits that there is a stress-induced decrease in adult hippocampal neurogenesis that leads to depression and that this decrease is reversed by ADs (Jacobs et al., 2000). The neurotrophic theory is based upon studies showing opposite effects of stress and ADs on expression of certain neurotrophins in brain (Duman et al., 1997, Duman and Monteggia, 2006). Brain derived neurotrophic factor (BDNF) is the most widely studied neurotrophin in this regard. Along with BDNF, its receptor, Tropomyosin related kinase B (TrkB), has also been studied. The neurotrophic and neurogenesis theories are linked; increased neurotrophin expression caused by ADs may block or reverse stress-related neuronal loss (Duman and Monteggia, 2006).

To some extent, all these theories rest upon effects produced by ADs. Although ADs are effective, about 15-30% of depressed patients do not respond to multiple treatments (Little, 2009) and are diagnosed as having treatment resistant depression (TRD); its occurrence demands better therapeutic strategies. Vagal Nerve Stimulation (VNS) therapy has been approved for treating TRD in several countries including the United States. Studies show promising results with VNS in TRD patients, particularly after treatment for 12 weeks, even persisting for a year or two (Schlaepfer et al., 2008, Berry et al., 2013). Previously, we reported that serotonergic and/or noradrenergic systems are involved in the behavioral effects of VNS (Furmaga et al., 2011). We also found that acute or chronic VNS treatment promotes phosphorylation of hippocampal TrkB (Furmaga et al., 2012) and that K252a, a non-specific tyrosine kinase inhibitor (Koizumi et al., 1988, Tapley et al., 1992), blocked TrkB phosphorylation caused by either acute VNS or desipramine (DMI) treatments (Furmaga et al., 2012). To extend these results, we first investigated the role of TrkB in the behavioral effects of VNS, using K252a, with the forced swim test (FST) providing a measure of antidepressant-like effects (Porsolt et al., 1978) and the novelty suppressed feeding test (NSFT) a measure of anxiolytic-like effects (Bodnoff et al., 1988).

As mentioned, AD drugs activate TrkB by phosphorylation at Y705, within the autophosphorylation catalytic domain of TrkB and also at Y816, which leads to activation of the PLC- γ 1 pathway (Saarelainen et al., 2003, Rantamaki et al., 2007, Furmaga et al., 2012). However, only acute or chronic VNS causes phosphorylation at Y515, linked to Ras/MAPK and PI3K pathways (Furmaga et al., 2012, Carreno and Frazer, 2014). It is currently not certain if AD-induced TrkB activation is due to BDNF release, or to a different neurotrophin

or is independent of neurotrophins (Rantamaki and Castren, 2008). In conditional forebrain BDNF knockout mice, treatment with imipramine still causes phosphorylation of TrkB at Y816 (Rantamaki et al., 2011). Also, BDNF *in vitro* and *ex vivo* causes phosphorylation at all three sites (Middlemas et al., 1994, Yuen and Mobley, 1999, Huang et al., 2008, Di Lieto et al., 2012). Based on these reports, one may hypothesize that AD-induced TrkB activation is not ligand-dependent whereas that by VNS may be. Therefore, we tested whether activation of TrkB in response to DMI and VNS treatments is ligand-dependent, by using a recombinant human TrkB-Fc chimera (or TrkB-Fc) consisting of the ligand-binding domain of TrkB with the Fc region of human IgG1. TrkB-Fc acts as a false receptor and sequesters endogenous neurotrophins (both BDNF and NT-4/5) with high potency and specificity as shown *in vitro* (Shelton et al., 1995) and *in vivo* (Binder et al., 1999, Gustafsson et al., 2003).

2. Experimental procedures

2.1 Animals

Adult male Sprague-Dawley rats (250-400 g, ~ 8 weeks old upon arrival, Harlan, Houston, TX) were group-housed on a 14:10 h light/dark cycle (lights on at 0700 h) and given one week to acclimatize before any procedures. Food and water were provided *ad libitum*, unless noted otherwise. Animals were isolated after surgery. All procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the local Institutional Animal Care and Use Committee (IACUC).

2.2 Antidepressant drug administration

2.2.1 Acute—Desipramine hydrochloride (DMI, 15 mg/kg, SC) or vehicle (sterile physiological saline) was administered 23.5 and 1 h before testing (Figure 1C).

2.2.2 Chronic—DMI (10 mg/kg/day, IP) or vehicle (distilled water) was delivered via osmotic minipumps (2.5 μ l/h, Model 2ML4, DURECT Corp., Cupertino, CA) as described by Furmaga et al. (Furmaga et al., 2011) for up to 24 days (Figure 1A).

Doses of DMI are expressed as free-base.

2.3 Vagal Nerve Stimulation

The surgery was performed as described by us previously (Cunningham et al., 2008, Furmaga et al., 2011), using stimulators generously provided by Cyberonics, Inc. (Houston, TX). Animals were allowed one-week recovery time before further procedures. Stimulation parameters used are similar to those used initially in clinical settings (Rush et al., 2005) and were as follows: current, 0.25 mA; frequency, 20 Hz; pulse width, 0.25 msec; duty cycle, 30 sec ON, 300 sec OFF (Furmaga et al., 2011). When given acutely, VNS was given 3 times for 2 h each time starting at -23.5, -6.5 and -2.5 h prior to testing in the FST (Cunningham et al., 2008). When given chronically, VNS was administered for periods up to 24 days using the stimulation parameters described above. Stimulators were turned off 30 min prior to the forced swim test (FST), switched on 5 min post-FST and turned off again before tissue

collection. To avoid confounding effects from handling, the same procedure was carried out with the Sham animals except that the current setting remained at 0 mA.

2.4 Stereotaxic surgery for intracerebroventricular (ICV) administration

Rats were anesthetized with ketamine and medetomidine (75 mg/kg and 0.5 mg/kg, respectively, IM) and placed onto a stereotaxic frame. Guide cannulae (C313G, 22G, Plastics One, USA) were implanted unilaterally targeting the lateral ventricle (from skull: AP −0.8, ML −1.4, DV −3.5; Paxinos and Watson (Paxinos and Watson, 1986)). Cannulae were cemented to the skull and capped using dummies (C313DC, Plastics One). Animals were allowed one-week recovery time before further procedures. K252a (1 µg in 1 µl dimethyl sulfoxide or DMSO, 0.25 µl/min over 4 min, Calbiochem, San Diego, CA) was administered to freely moving rats via injectors (C313I, 28G, Plastics One) that fit into guide cannulae with a 1 mm projection. Injectors were left in place for 5 min post-injection to allow diffusion. The K252a administration protocol was adapted from the description by Li et al. (2011).

In the acute study with DMI, K252a was given 2 h prior to each injection of the drug (Figure 1C). With acute VNS, it was administered at time points shown in Figure 1D. In the chronic treatment paradigms, K252a was administered 2 h before turning on the stimulators on Day 0 to VNS-treated animals and every other day, starting on Day 1 until the end of the study to both VNS and DMI-treated animals and respective controls (Figures 1A and 1B).

2.5 Behavioral tests

2.5.1 Novelty suppressed feeding test (NSFT)—The NSFT was performed on Day 10 (see Figures 1A and 1B) and carried out as originally described by Bodnoff et al. (1988) and by us previously (Furmaga et al., 2011, Roth et al., 2012). Rats were food deprived for 24 or 48 h prior to testing. The original description for the NSFT by Bodnoff et al. (1988) used a 48-h food deprivation and this is what we used successfully in our previous experiments (Furmaga et al., 2011). In this cohort of DMI-treated animals, in preliminary experiments we found such food deprivation to cause very short latencies. As the latencies were so low in the control rats, in these preliminary experiments DMI had little effect. Upon decreasing the food deprivation to just one day the latencies increased substantially in the control animals, which allowed the effect of DMI to be seen. Rats were then individually placed into the corner of a novel black Plexiglas open field (100 × 100 × 40 cm) facing the center where food pellets were placed. The latency to begin feeding and the amount of food consumed during the 12-min test were recorded. During analysis of all behavioral tests, raters were blind to treatment conditions.

To account for effects of treatment/s on appetite, food consumption was monitored during the test and in the home cage for 30 min post-testing. Total food consumed was determined by adding the amounts consumed during the test and in the home cage.

2.5.2 Forced swim test (FST)—The FST was performed on Day 22 (see Figures 1A and 1B) and carried out essentially as described by Detke et al. (1995) and by us previously (Furmaga et al., 2011). For chronic studies, there was no training session as effects of

chronic AD treatment in the FST are observed even in the absence of this session (Cryan et al., 2005, Furmaga et al., 2011). For acute studies, a 15-min training session was carried out 26 h prior to the 5-min test session. Rat behavior was recorded and scored using a time-sampling technique to rate the predominant behavior (climbing, swimming, immobility) over 5 s intervals (bins) over the 5-min testing period.

2.5.3 Analysis of locomotor activity in an open field—Ambulatory behavior of rats was examined on Day 18 of the chronic studies (Figures 1A and B) to account for potential treatment effects on this behavior. Rats were individually placed into a novel Plexiglas open field (50 cm × 50 cm × 40 cm). Total distance traveled was monitored and recorded by automated video tracking software, ANY-Maze (San Diego Instruments, CA). Distances traveled during the first 15 min are reported.

2.6 Tissue collection, protein estimation and Western blot analysis

For all groups, rats were briefly anesthetized by placing in a sealed cylinder containing gauze soaked with isoflurane under a mesh immediately before decapitation. Rats with stimulators were injected intracardially with 1 ml of 3 M KCl while still anesthetized to allow quick removal of the stimulating electrodes. After rapid decapitation, brains were removed and the left hippocampus dissected out. Sample preparation for Western blotting, blotting procedure, visualization and quantification of blots was carried out as described by Carreno et al. (2014). Samples were homogenized in lysis buffer (50mM Tris, 1 mM EDTA, 0.35% Sodium deoxycholate, 150 mM NaCl, 1% Igepal, 10% glycerol, 0.1% SDS with pH adjusted to 7.4 and supplemented with a cocktail of protease and phosphatase inhibitors), incubated on ice for 30 min and centrifuged (16,000 g for 20 min). Supernatants were collected and protein levels estimated using Bradford's assay (Bio-rad, USA). Proteins were then separated on a 7.5 or 10% SDS-PAGE gel, blotted onto a nitrocellulose membrane at 100 V for 1 h. Membranes were incubated overnight at 4°C using anti-pY705 TrkB (1:1000 in 2.5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST), Abcam, USA) or anti-TrkB (full length, 1:5,000 in 2.5% milk in TBST, Neuromics, USA). β -Actin (1:800,000 in 2.5% milk, Sigma, USA) was used as a loading control. Membranes were washed with TBST and incubated with horseradish peroxidase conjugated secondary antibodies (1:20,000 in 2.5% BSA in TBST, Sigma, USA). Densitometry analysis of immunoreactive bands was performed using ImageJ software (NIH, USA). Results were calculated as ratios of arbitrary densitometric units of phosphorylated tyrosine, Y705 to total non-phosphorylated TrkB protein, normalized by values for actin and expressed as a percentage of the control (Sham or vehicle) group.

2.7 Determination of DMI levels in serum

For the acute study, trunk blood was collected at the end of the experiment whereas for the chronic study, blood was collected by tail clip bleeding on Day 13. Tails were cleaned with an alcohol swab and allowed to dry before making a transverse section through the long axis approximately 2 mm from the tip with a sterile scalpel blade. Blood was collected into tubes by massaging the tail from the base to the tip. After blood collection, direct pressure was applied on the tail for a few minutes to facilitate hemostasis. Serum DMI levels were

determined by HPLC with a Waters spherisorb S5 CN column and UV detection at 214 nm in the laboratory of Dr. Martin Javors, Department of Psychiatry, UTHSCSA.

2.8 Studies involving TrkB-Fc chimera

An initial study was performed in naïve animals to determine the extent of distribution of TrkB-Fc (20 µg in 10 µl) at various time points post ICV administration. Rats were anesthetized with ketamine (75 mg/kg, IM) and medetomidine (0.5 mg/kg, IM). TrkB-Fc (20 µg in 10 µl, R&D Systems, USA) was injected into the right lateral ventricle (from skull: AP -0.8, ML -1.4, DV -4.0; Paxinos and Watson (1986)) via injectors (28G, Plastics One) at the rate of 1 µl/min; injectors were left in place for 5 min to allow for diffusion. Post-operative injections included penicillin, saline and atipamezole (1 mg/kg; Antisedan, Pfizer, USA) to reverse sedative effects of the anesthetic. Animals were perfused with 4% paraformaldehyde 15 min, 3 h or 8 h post TrkB-Fc administration. Brains were dissected and sectioned into 40-µ thick slices processed for immunohistochemical detection of TrkB-Fc carried out essentially as described by Gustafsson et al. (2003) with primary antibody (Hu-IgG, 1:10,000, goat polyclonal, Sigma, USA) and rabbit anti-goat secondary antibody (1:200, Invitrogen, USA) diluted in 2% normal rabbit serum in 0.25% Triton X-100 in PBS.

The experimental timeline (Figure 1E) was determined based on the distribution pattern of TrkB-Fc at various time points. VNS devices (surgically implanted one week prior to the experiment) were turned on 2 h after ICV administration of TrkB-Fc/PBS for a 2 h period and remained on during tissue collection (Figure 1E). Similarly, for the DMI/Vehicle groups, the drug (10 mg/kg, IP) or vehicle (sterile physiological saline) was injected 2 h after TrkB-Fc/PBS administration (Figure 1E).

Animals were rapidly decapitated 2 h after initiation of VNS or drug treatment (Figure 1E). The right hippocampus was dissected out and cut approximately midway into predominantly dorsal and ventral portions. The dorsal portion was processed for Western blotting as described earlier.

2.9 Statistical analysis

Two-way ANOVAs were performed for behavioral and Western blot analyses followed by planned comparisons using Fisher's Least Significant Difference (LSD) tests. Planned comparisons were between (1) the treatments (VNS or DMI) and respective controls and (2) the inhibitor (K252a or TrkB-Fc) and respective controls. The level of significance was set as $p < 0.05$ for all analyses. All analyses were carried out using GraphPad Prism 6 (GraphPad Software, Inc., CA).

3. Results

3.1 Chronic studies

There was no significant difference between levels of DMI in serum achieved in the absence or presence of K252a; hence values for all DMI-treated animals were combined, with the mean value ($\pm$ SEM) being 197 ± 46.8 ng/ml ($n = 17$).

To confirm that chronic administration of K252a inhibited TrkB phosphorylation, hippocampal tissue was collected in the same cohort of animals that were used for behavioral analysis and analyzed by Western blot analysis. As anticipated, chronic treatment with DMI or VNS produced a significant increase in phosphorylation at Y705, which was blocked by administration of K252a. K252a treatment alone had no effect on phosphorylation of Y705 (Figure 2). Two-way ANOVA revealed a significant main effect of DMI [$F(1,18) = 6.36, p < 0.05$] and K252a-treatments [$F(1,18) = 17.09, p < 0.001$] and a significant interaction between the two treatments [$F(1,18) = 6.14, p < 0.05$]. Planned comparisons showed a significant effect of DMI within the DMSO-treated groups ($p = 0.002$) but not within K252a-treated groups. Also, there was no significant difference between vehicle-treated animals in the DMSO and K252a groups (Figure 2A). Two-way ANOVA revealed a significant interaction between VNS and K252a-treatments [$F(1,43) = 10.1, p < 0.005$]. Planned comparisons showed a significant effect of VNS treatment within the DMSO-treated groups ($p = 0.004$) but not in animals treated with K252a. Further, there was no significant difference between Sham-treated animals in the DMSO and K252a groups (Figure 2B). Thus, K252a blocked DMI- and VNS-induced increases in phosphorylation at Y705 on TrkB.

3.1.1 Effects of TrkB inhibition on behavioral responses to DMI and VNS treatments in the NSFT

As ADs produce an anxiolytic-like effect in the NSFT only after chronic administration (Bodnoff et al., 1988, Santarelli et al., 2003), we assessed the role of TrkB in the anxiolytic-like effects of chronic DMI and VNS treatments on day 10. Results for latencies to feed are shown in Figure 3. Both treatments produced an anxiolytic-like effect as indicated by a significant reduction in latency to feed in the NSFT (Figure 3). K252a blocked the anxiolytic-like effect of VNS treatment. However, it did not block the anxiolytic-like effect of DMI, i.e. DMI significantly reduced latency to feed even in animals treated with K252a. K252a alone had no effect on latency to feed (Figure 3). Two-way ANOVA revealed a significant main effect of DMI treatment [$F(1,20) = 42.13, p < 0.0001$]. Planned comparisons revealed a significant effect of DMI within DMSO-treated animals ($p = 0.0005$) and within K252a-treated animals ($p < 0.0001$). There was no significant difference between latencies for vehicle-treated animals in the DMSO and K252a groups. Two-way ANOVA revealed a significant interaction between VNS treatment and the inhibitor [$F(1,50) = 5.46, p < 0.05$]. Planned comparisons revealed a significant effect of VNS in reducing latency ($p = 0.038$) in animals injected with DMSO but not in those treated with K252a. There was no difference in latency between Sham-treated animals in the DMSO and K252a groups. Thus, K252a blocked the anxiolytic-like effect of VNS but not that of DMI, when these treatments were given chronically.

In the DMI/Vehicle groups, neither drug nor inhibitor treatment had any significant effects on home cage or total food consumption (data not shown).

In the VNS/Sham groups, Two-way ANOVA revealed a significant effect of K252a treatment on home cage [$F(1,49) = 12.01, p < 0.005$] and on total [$F(1,49) = 4.36, p < 0.05$] food consumption. However, this increased food consumption by the K252a-treated animals did not result in a reduced latency in these groups (Figure 3). Also, VNS treatment itself did

not have a significant effect on food consumption; hence the interpretation of our results is not confounded by probable changes in appetite (data not shown).

3.1.2 Effects of TrkB inhibition on behavioral responses of DMI and VNS

treatments in the FST—As anticipated, an antidepressant-like effect of chronic administration of DMI was observed in the FST as indicated by an increase in climbing behavior and decrease in immobility (Figure 4). However, K252a did not block this effect. Further, K252a alone did not alter immobility, climbing or swimming behaviors. Two-way ANOVA revealed a significant main effect of DMI on immobility [$F(1,29) = 23.66$, $p < 0.0001$]. Planned comparisons showed a significant effect of DMI in DMSO-treated ($p = 0.0002$) and in K252a-treated ($p = 0.012$) animals. Two-way ANOVA revealed a significant main effect of DMI on climbing behavior [$F(1,29) = 53.66$, $p < 0.0001$]. Planned comparisons showed a significant effect of DMI on climbing behavior within DMSO-treated ($p < 0.0001$) and K252a-treated ($p < 0.0001$) animals. Two-way ANOVA revealed a significant main-effect of DMI on swimming behavior [$F(1,29) = 9.31$ ($p < 0.005$)]. Planned comparisons showed a small but significant decrease in swimming behavior caused by DMI in the K252a-treated animals ($p = 0.013$).

Previously we found that chronic VNS (14 days) produces an antidepressant-like effect in the FST (Furmaga et al., 2011). However, we did not see this effect in animals that were simultaneously treated with VNS (22 days) and repeated ICV injections of DMSO (data not shown). As there was no effect of VNS treatment itself; we could not test the role of TrkB with respect to chronic VNS administration in the FST.

3.1.3 Effects of VNS, DMI and/or inhibitor treatments on locomotor activity in an open field—Distances moved (Mean $\pm$ SEM, m) during the first 15 min by the DMI/Vehicle groups in the open field were as follows: DMSO/Vehicle: 25.1 ± 2.79 ; DMSO/DMI: 18.2 ± 0.75 ; K252a/Vehicle: 25.77 ± 2.08 ; K252a/DMI: 19.94 ± 2.9 .

While there was a significant main effect of DMI [$F(1,15) = 7.159$, $p < 0.05$], planned comparisons did not reveal any significant differences between groups. DMI does show a trend towards decreased locomotor activity in the DMSO- as well as K252a- treated groups. This effect of DMI on ambulatory behavior has been reported previously (Wongwitdecha et al., 2006). However, as we do not observe an increase in immobility in the FST with DMI, the DMI-induced *reduction* of locomotor activity in the open field does not appear to be a confounding factor for the ability of the K252a treatment to block its effects in the FST.

Distances moved (Mean $\pm$ SEM, m) by the VNS/Sham groups were as follows: DMSO/Sham: 22.32 ± 5.33 ; DMSO/VNS: 25.37 ± 1.13 ; K252a/Sham: 29.85 ± 5.64 ; K252a/VNS: 21.22 ± 4.31 . There were no significant differences in locomotor activity among these groups.

3.2 Acute studies

In light of our results showing no effect of TrkB inhibition on the behavioral effects of chronic treatment with DMI in rats, its effect on acute DMI treatment was also evaluated. This study was carried out as shown in Figure 1C. Immediately after the FST, trunk blood

was collected and serum DMI levels measured. There was no significant difference between levels achieved in the absence or presence of K252a; hence the values for all DMI-treated animals were combined. Two injections of DMI (15 mg/kg, SC) administered 23.5 and 1 h prior to testing resulted in serum DMI levels of 680 ± 37.2 ng/ml (Mean $\pm$ SEM, $n = 8$).

3.2.1 Effect of K252a administration on the antidepressant-like effect of acute DMI and VNS treatments in the FST—As expected, DMI had an antidepressant-like effect indicated by a significant decrease in immobility and increase in climbing. K252a alone did not affect behavior. However, in contrast to the results seen with its chronic administration, K252a blocked the decrease in immobility and increase in climbing caused by DMI (Figure 5A). Two-way ANOVA for immobility scores revealed a significant main effect of DMI treatment [$F(1,32) = 5.40$, $p < 0.05$]. Planned comparisons revealed a significant difference between immobility scores for animals treated with DMI and saline within the DMSO-treated groups ($p = 0.013$). However, there was no significant difference between immobility scores for animals treated with DMI and saline within the K252a-treated group. Immobility scores for vehicle-treated animals also did not differ between DMSO and K252a treated groups. Two-way ANOVA for climbing scores revealed a significant main effect of DMI [$F(1,32) = 16.17$, $p < 0.0005$] and K252a [$F(1,32) = 4.35$, $p < 0.05$] treatments. Planned comparisons revealed a significant difference between climbing scores for animals treated with DMI and saline (vehicle) within the DMSO-treated groups ($p = 0.0004$). However, there was no significant difference between climbing scores for animals treated with DMI and saline within the K252a-treated group. Climbing scores for vehicle-treated animals also did not differ between DMSO and K252a treated groups. As expected, two-way ANOVA for swimming behavior showed no significant main effects or interaction with DMI and K252a.

For the chronic VNS experiment, the combination of stimulator and cannula may have caused problems in the FST such that the effects of VNS on immobility and swimming were quite variable. Nevertheless, we thought it useful to see if we could obtain positive results in the FST after acute VNS treatment and, if so, whether K252a, administered via an ICV cannula, would block such effects (Figure 5B). VNS did not decrease immobility significantly but it did cause a small but significant increase in swimming behavior that was blocked in the presence of K252a. Two-way ANOVA for immobility showed no significant main effects. Two-way ANOVA for swimming showed a significant main effect of VNS treatment [$F(1,28) = 5.04$, $p < 0.05$]. Planned comparisons showed a significant difference between DMSO-Sham and DMSO-VNS groups ($p = 0.031$) whereas K252a-Sham and K252a-VNS groups did not differ from each other. Two-way ANOVA for climbing behavior showed a significant main effect of inhibitor treatment [$F(1,28) = 5.70$, $p < 0.05$]. However, planned comparisons did not reveal any significant differences between groups. Thus, K252a blocked acute behavioral effects of both VNS and DMI whereas it only blocked a chronic behavioral effect of VNS but not that of DMI.

3.3 Effect of TrkB-Fc pretreatment on TrkB activation by DMI and VNS treatments

Based on the immunohistochemical detection of its distribution (Figure 6A), we anticipated that TrkB-Fc would be present primarily in the dorsal hippocampus between 2 and 4 h-post

ICV administration. Hence, TrkB-Fc (or sterile PBS) was injected 2 h prior to DMI (or Vehicle) injections or VNS (or Sham) treatment, and dorsal hippocampal tissue collected 2 h after this time-point as shown schematically in Figure 1E. The chimera blocked the ability of either DMI (Figure 6B) or VNS (Figure 6C) to phosphorylate TrkB. Two-way ANOVA revealed a significant interaction between DMI treatment and TrkB-Fc for phosphorylation of TrkB [$F(1,24) = 4.79$, $p < 0.05$]. Planned comparisons revealed a significant effect of DMI within the PBS-treated animals ($p = 0.023$). However, there was no significant effect of DMI within groups treated with TrkB-Fc. Further, there was no difference in Y705 phosphorylation between saline-treated animals in the PBS- and TrkB-Fc groups (Figure 6B). Similarly, for VNS, two-way ANOVA revealed a significant interaction between VNS and TrkB-Fc treatments for TrkB phosphorylation [$F(1,30) = 8.52$, $p < 0.01$]. Planned comparisons revealed a significant effect of VNS within the PBS-treated animals ($p = 0.022$). However, there was no significant effect of VNS within the groups treated with TrkB-Fc. Further, there was no difference in Y705 phosphorylation between sham-treated animals in the PBS- and TrkB-Fc groups (Figure 6C).

4. Discussion

There are several main findings of this study. First, the behavioral effects of chronic DMI treatment in the FST and in the NSFT were not blocked by K252a; however, its acute effect in the FST was blocked by this inhibitor of TrkB phosphorylation. By contrast, the anxiolytic-like effect of chronic VNS treatment in the NSFT was blocked by K252a, as was its acute effect in the FST. Further, from the results of the second set of experiments, it may be inferred that TrkB activation that occurs in the dorsal hippocampus in response to either acute DMI or VNS administration requires ligand binding as TrkB-Fc, a scavenger for endogenous neurotrophin ligands (BDNF and NT-4/5), blocked both DMI- and VNS-induced phosphorylation of TrkB.

K252a failed to block the behavioral effects of chronic DMI but blocked the effect of acute DMI treatment in spite of serum DMI concentrations being much higher in rats treated acutely with DMI. Also, chronic administration of K252a was able to block the effect of VNS in the NSFT even though given essentially identically to how it was given in the rats treated with DMI (see Figures 1A and B).

Previously, we demonstrated that acute (2 h) or chronic (14 days) treatments with DMI or VNS induces phosphorylation of TrkB and that pretreatment with K252a blocked acute DMI and VNS-induced TrkB phosphorylation in the hippocampus (Furmaga et al., 2012). In the present study, we now show that repeated administration of K252a also blocks chronic DMI- or VNS-induced phosphorylation of hippocampal TrkB. In spite of this blockade, it did not block the chronic behavioral effects of DMI.

Despite the compelling evidence supporting a link between BDNF and AD drug action, there are also inconsistent findings with respect to effects of such drugs on hippocampal levels of mRNA for BDNF (Nibuya et al., 1996, Russo-Neustadt et al., 1999, Conti et al., 2002, Miro et al., 2002, Altieri et al., 2004) or protein levels (Altar et al., 2003, Xu et al., 2003, Jacobsen and Mork, 2004). In addition, after three weeks of treatment with DMI,

Jacobsen and Mork (2004) report no changes in mRNA for TrkB in either the hippocampus or frontal cortex. Although these inconsistencies may be due to differences in animal strains, drug treatment paradigms, doses and durations of treatment, they point out that the involvement of neurotrophins in the effects of AD drugs is far from proven. Our results also demonstrate that at least for chronic treatment with DMI, TrkB activation does not seem to be involved in its behavioral effects. However, in line with the neurotrophin hypothesis (Duman and Monteggia, 2006, Kozisek et al., 2008), our results with acute treatment with DMI and VNS in the FST in rats suggest a critical role for TrkB activation in the antidepressant-like effects of these treatments and are consistent with the lack of effect of drugs in the FST in BDNF heterozygote knockout mice (Saarelainen et al., 2003, Monteggia et al., 2004) and in mice over-expressing the dominant-negative truncated form of the receptor in neurons of the hippocampus and cortex (Saarelainen et al., 2003).

K252a belongs to the K252 family of alkaloid protein kinase inhibitors and was initially described as an inhibitor of protein kinase C (PKC) (Kase et al., 1986). However, it can inhibit Trk-dependent actions at concentrations lower than those necessary to block PKC-dependent effects (Koizumi et al., 1988). A subsequent study established that K252a can inhibit the tyrosine kinase activity of Trk receptors (Tapley et al., 1992). Most frequently, it has been described as a potent and relatively selective inhibitor of tyrosine kinase activity associated with trk receptors with little activity against other tyrosine kinases such as those for epidermal growth factor, Src and insulin (Berg et al., 1992, Nye et al., 1992, Ohmichi et al., 1992, Tapley et al., 1992). It has been used widely to antagonize biological functions of BDNF (Nye et al., 1992) and Trk receptors *in vitro* (Bradley and Sporns, 1999) and *in vivo* (Pinnock et al., 2010). Inhibition of Trk receptors by K252a occurs via interaction with the intracellular kinase domain and not by association with the ligand or ligand-binding site. Local application of K252a has been used successfully to block the antidepressant-like effects of BDNF that is exogenously administered into the hippocampus (Shirayama et al., 2002).

The NSFT was used to investigate if TrkB activation was required for the anxiolytic effect of VNS and DMI treatments. This test involves hyponeophagia or inhibition of feeding produced by exposure to novelty. It displays both predictive and construct validities for the anxiolytic effects of drug treatments (Dulawa and Hen, 2005). The effects of AD drugs in this test are consistent with their effects in humans, where an anxiolytic response is seen only when the drugs are administered chronically (Bodnoff et al., 1988, Santarelli et al., 2003, Dulawa et al., 2004). AD treatments are used to treat anxiety as well as depression and almost all classes of compounds used to treat depression also reduce anxiety (see Dulawa and Hen, 2005). Further, anxiety and depression are inter-related phenomena in humans (Helmuth, 2003, Morilak and Frazer, 2004). Although K252a abolished the anxiolytic-like effect of VNS treatment, the anxiolytic-like effect of DMI treatment persisted in spite of K252a administration. Thus, the anxiolytic-like effect of chronic VNS seems to involve activation of TrkB whereas that of DMI may not.

The FST has been widely used to identify potential AD compounds (Porsolt et al., 1977, Cryan et al., 2002, Slattery and Cryan, 2012). We have previously shown that acute DMI and VNS treatments have antidepressant-like effects in this test (Cunningham et al., 2008). Here,

we now show that K252a abolished these antidepressant-like effects produced by acute DMI or VNS treatments. It may be inferred from such results that acute effects of DMI in the FST require activation of TrkB whereas this is not the case for effects seen after its chronic administration.

As drugs that are motor-impairing may confound results in this test (Slattery and Cryan, 2012), locomotor activity was monitored and there were no significant effects of AD treatment and/or inhibitor treatment.

In a study connecting TrkB to neurogenesis and AD efficacy, Li et al. (2008) showed that ablation of TrkB in hippocampal neural progenitor cells in the dentate gyrus caused impaired proliferation and neurogenesis of these cells and rendered the mice insensitive to chronic AD treatment in depression and anxiety-like paradigms. By contrast, mice lacking TrkB only in the differentiated dentate gyrus neurons showed normal neurogenesis and responded normally to chronic ADs. In an attempt to reconcile the paradoxical findings related to BDNF/TrkB and AD action, Monteggia et al. (2007) have speculated that perhaps AD efficacy requires a certain level of baseline BDNF whereas large increases in this neurotrophin are not needed for AD efficacy. This may explain why a substantial decrease in BDNF baseline levels may abolish AD efficacy while impaired upregulation of BDNF does not have as strong an effect. Overall, it appears that the extent of TrkB's role in the behavioral effects of classical ADs may be dependent on factors such as brain region, duration of treatment, animal model (stressed versus naïve) and species. As mentioned, our results suggest that TrkB activation is required for the behavioral effects of acute but not chronic treatment with DMI. However, we cannot rule out the possibility that TrkB activation may be involved in the actions of chronic DMI in other behavioral models of depression, such as those involving chronic stress paradigms or social defeat and measures of anhedonia.

Previously, by using selective lesions of central noradrenergic or serotonergic systems, the anxiolytic-like and antidepressant-like effects of chronic DMI treatment were shown to be dependent on an intact noradrenergic system and the anxiolytic-like effects of VNS were found to require an intact noradrenergic and serotonergic system (Furmaga et al., 2011). Here we show that the anxiolytic-like effect of chronic DMI treatment may not require TrkB phosphorylation whereas that of VNS does. Overall, it seems as though VNS may employ several different mechanisms for its beneficial effects, e.g., noradrenergic, serotonergic, neurotrophins such as BDNF/NT-4/5 whereas behavioral effects of chronic treatment with a drug such as DMI may involve primarily noradrenergic neurons. Whether these systems are linked or produce useful effects through independent pathways is an important area for future research. If independent, as perhaps suggested by our collective observations with chronic DMI treatment, then treatments for TRD such as VNS may be more effective due to the recruitment of multiple biological systems for its effects (Blier et al., 2010, Hayley and Litteljohn, 2013).

Interestingly, ketamine, a drug presently in the limelight for treating TRD, transiently increases phosphorylation of TrkB after a single injection in rodents (Autry et al., 2011, Duman and Aghajanian, 2012). As discussed (Shah et al., 2014), both VNS and ketamine

modulate signaling pathways downstream of TrkB, such as the mammalian target of rapamycin (mTOR) pathway within the hippocampus indicated by increased phosphorylation of p70S6 kinase, in addition to AKT and ERK (Autry et al., 2011, Duman and Aghajanian, 2012, Carreno and Frazer, 2014). More importantly, these effects are not seen with chronic administration of traditional antidepressants (Carreno and Frazer, 2014).

As mentioned earlier, DMI and other AD drugs induce TrkB phosphorylation at Y705 and Y816 (Saarelainen et al., 2003, Rantamaki et al., 2007, Furmaga et al., 2012, Carreno and Frazer, 2014). However, VNS-induced TrkB phosphorylation is observed at an additional site, Y515 (Furmaga et al., 2012) and it also activates signaling molecules downstream of Y515 whereas DMI does not (Carreno and Frazer, 2014). *In vitro* studies, using embryonic fibroblast cells, show that BDNF causes phosphorylation at all three sites, in a time and dose-dependent manner (Yuen and Mobley, 1999) and activates signaling cascades downstream of these sites (Middlemas et al., 1994, Qian et al., 1998, Yuen and Mobley, 1999). NT-4/5 also seems to have the same effect on TrkB phosphorylation (Yuen and Mobley, 1999). Further, a more recent *ex vivo* study using adult cortical and hippocampal mouse brain lysates showed that BDNF can induce TrkB phosphorylation at Y816 and Y515 in a cell-free kinase activity assay (Di Lieto et al., 2012). Hence, the lack of antidepressant-induced phosphorylation at Y515 led us to hypothesize that DMI-induced phosphorylation may perhaps not be ligand-dependent. This hypothesis was strengthened by the observation by Rantamaki et al. (2011), that conditional knockout mice lacking BDNF in the forebrain still showed phosphorylation at Y816 in response to acute imipramine treatment, although to a lesser extent than in wild-type mice. Further, there is evidence of activation of TrkB independent of binding by neurotrophins, either by transactivation (Lee et al., 2002, Nagappan et al., 2008, Boulle et al., 2012) or basal activity induced by increased receptor density within intracellular vesicles and lipid rafts of the plasma membrane (Swift et al., 2011).

To address whether DMI and/or VNS-induced TrkB phosphorylation was ligand independent, we pre-administered TrkB-Fc and assessed phosphorylation of Y705. To the best of our knowledge, this scavenger chimera has not been used in the context of AD-related BDNF/TrkB signaling. From our results, it may be inferred that both DMI- and VNS- induced TrkB phosphorylation are ligand-dependent, as the phosphorylation of Y705 caused by both was blocked by TrkB-Fc. Therefore, the reason for the lack of phosphorylation at Y515 with DMI remains unclear.

There are several limitations of our study. The practical limitations are due to the size of the VNS stimulator device (weighing approximately 16 g). This prevents us from using smaller animals such as mice, which could allow more sophisticated genetic approaches to study some pertinent questions. In addition, even with rats it appears from our FST experiments that the presence of the device along with further manipulations (such as cannulae for inhibitor administration) may contribute to physical constraints in certain behavioral tests.

Further, the pharmacological approach can be a limitation since as mentioned earlier K252a is a non-specific tyrosine kinase inhibitor. Although we have shown that it blocks TrkB in our studies, we cannot rule out the possibility that our results may be due to the effects of

K252a on a tyrosine kinase other than TrkB even though K252a has little activity against tyrosine kinases other than trk receptors (Berg et al., 1992, Nye et al., 1992, Ohmichi et al., 1992, Tapley et al., 1992). Further, K252a (Sondell et al., 1999) does not block the neurotrophic effects of vascular endothelial growth factor through its tyrosine kinase receptor. Nevertheless, further studies will have to be carried out with other well-characterized pharmacological agents or genetic manipulations that specifically target TrkB, in order to confirm the role of TrkB in the chronic behavioral effects of antidepressant treatments.

Further, TrkB-Fc not only binds BDNF but also NT-3 and NT-4/5 (Shelton et al., 1995). So it is possible that these other neurotrophins, in addition to BDNF, are involved in the effects observed. However, BDNF expression is more abundant than NT-3 or NT-4/5 in the hippocampus (Friedman et al., 1998).

In conclusion, we have shown that the antidepressant-like effect of acute treatment with DMI, a traditional AD, in the FST requires activation of TrkB. By contrast, the effects of chronic treatment with DMI in this test as well as in the NSFT do not require TrkB activation. However, both acute and chronic treatments with VNS, a therapeutic option for TRD, require activation of TrkB for antidepressant-like and anxiolytic-like effects, respectively. In addition, we have shown that phosphorylation of TrkB in the dorsal hippocampus by acute DMI and VNS is ligand-dependent.

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Abbreviations

BDNF	Brain derived neurotrophic factor
TrkB	Tropomyosin related kinase B
AD	Antidepressant
TRD	Treatment Resistant Depression
VNS	Vagal Nerve Stimulation
DMI	Desipramine
IP	Intraperitoneal
FST	Forced Swim Test
NSFT	Novelty Suppressed Feeding Test
Y705	Tyrosine residue 705
Y816	Tyrosine residue 816
PLC-γ1	Phospholipase C- gamma1

Y515	Tyrosine residue 515
MAPK	Mitogen-activated Protein Kinase
PI3K	Phosphoinositide 3-Kinase
NT-4/5	Neurotrophin-4/5
ICV	Intracerebroventricular
DMSO	Dimethyl Sulfoxide
TBST	Tris-Buffered Saline with 0.1% Tween 20
BSA	Bovine Serum Albumin
PBS	Phosphate-Buffered Saline
HPLC	High-Performance Liquid Chromatography
ANOVA	Analysis of Variance
LSD	Least Significant Difference
SEM	Standard Error of the Mean
Src	SRC proto-oncogene

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Highlights

- TrkB activation by chronic VNS is necessary for its anxiolytic-like effect.
- TrkB activation by chronic desipramine is not critical for its behavioral effects.
- TrkB activation is required for the behavioral effects of acute VNS and desipramine.
- Phosphorylation of TrkB by VNS and desipramine is ligand (BDNF/ NT-4/5)-dependent.

A. Chronic DMI

B. Chronic VNS

C. Acute DMI

D. Acute VNS

E. Experiment involving TrkB-Fc

Figure 1. Experimental paradigms for chronic studies with DMI (A) and VNS (B) treatments and K252a

Animals were allowed one week of recovery time after surgeries for implantation of VNS devices and stereotaxic surgeries for implantation of cannulae into the lateral ventricle. Osmotic pumps containing desipramine (DMI, 10 mg/kg/day, IP) or vehicle were implanted on Day 0 (A) whereas VNS devices implanted in advance were turned 'on' in animals belonging to the 'VNS' group, on Day 0 (B). In all groups, the NSFT was performed on Day 10, locomotor activity tested on Day 18, the FST performed on Day 22. K252a (1 μ g in 1 μ l DMSO, ICV) or vehicle (DMSO, 1 μ l, ICV) was initially administered 2 h before turning on

stimulators on Day 0 and every other day, starting from Day 1 till the end of the study. About 24 h after the last behavioral test, K252a was injected again and hippocampal tissue collected approximately 24 h post-injection. Experimental paradigms for acute studies with DMI (C) and VNS (D) treatments and K252a. The paradigm depicts time-points at which desipramine (DMI, 15 mg/kg, SC) or saline were administered, 23.5 and 1 h prior to testing in the FST. The training session was carried out 26 h prior to testing. K252a (1 µg in 1 µl DMSO, ICV) or vehicle (DMSO, 1 µl, ICV) was administered into the lateral ventricle, 2 h prior to each DMI/saline injection, via cannulae stereotactically implanted one week prior to the training day (C). Paradigm depicting time-points at which VNS (3 times for 2 h each starting at -23.5, -6.5 and -2.5 h prior to testing) and K252a or vehicle treatments (once 2 h before the first VNS session and the second time, immediately before the second VNS session) were administered (D). Experimental paradigm for studies involving the TrkB-Fc chimera (E). TrkB-Fc (20 µg in 10 µl, ICV) or vehicle was administered 4 h prior to tissue collection. DMI (10 mg/kg, IP) or vehicle was injected in the 'DMI' or 'Vehicle' groups whereas VNS devices implanted one week prior to experimentation were turned 'on' in animals belonging to the 'VNS' group, 2 h after TrkB-Fc administration, for a duration of 2 h after which animals were sacrificed and hippocampal tissue collected for molecular analysis by Western blotting (E).

Figure 2. Effect of repeated ICV administration of K252a on TrkB phosphorylation at Y705 in the hippocampus induced by chronic DMI (10 mg/kg/day, IP) (A) and VNS (B) treatments

The ability of chronic DMI or VNS treatment to induce phosphorylation of TrkB at Y705 was abolished by repeated administration of K252a (1 µg in 1 µl DMSO, ICV).

PhosphoY705 (pY705) values are normalized against Total TrkB values (n for DMSO/Vehicle = 6, DMSO/DMI = 5; K252a/Vehicle = 6; K252a/DMI = 5; DMSO/Sham = 12; DMSO/VNS = 11; K252a/Sham = 12; K252a/VNS = 12) *Significantly different from relevant control, $p < 0.05$ (Two-way ANOVA followed by planned comparisons). Digital images represent immunoreactive bands for pY705 TrkB that aligns with total TrkB at ~120-145 kDa, Total TrkB (~120-145 kDa) and beta-Actin (~50 kDa).

Figure 3. Effect of repeated ICV administration of K252a on the anxiolytic-like effects of chronic (10 days) DMI (10 mg/kg/day, IP) (A) and VNS (B) treatments on latency to feed in the novelty suppressed feeding test

K252a (1 μ g in 1 μ l DMSO) or DMSO was injected (ICV) every other day throughout the duration of DMI or VNS treatment (n for DMSO/Vehicle = 7, DMSO/DMI = 6; K252a/Vehicle = 6; K252a/DMI = 5; DMSO/Sham = 15; DMSO/VNS = 12; K252a/Sham = 13; K252a/VNS = 14). *Significantly different from relevant control value, $p < 0.05$ (Two-way ANOVA followed by planned comparisons using Fisher's LSD test)

Figure 4. Effect of repeated ICV administration of K252a on the antidepressant-like effects of chronic DMI (10 mg/kg/day, IP) treatment in the forced swim test

K252a (1 μ g in 1 μ l DMSO) or DMSO was injected (ICV) every other day throughout the duration of DMI treatment. Within the DMSO-treated animals, DMI significantly decreased immobility (left) and increased climbing behavior (right). This effect of DMI persisted in the K252a-treated animals. There was a small but significant reduction in swimming behavior by DMI in K252a-treated animals (n for DMSO/Vehicle = 8, DMSO/DMI = 8; K252a/Vehicle = 9; K252a/DMI = 8). *Significantly different from relevant control value, $p < 0.05$ (Two-way ANOVA, followed by planned comparisons using Fisher's LSD test)

Figure 5. Effect of ICV administration of K252a on the antidepressant-like effects of acute DMI (15 mg/kg, SC) (A) and VNS (B) treatments in the forced swim test

K252a (1 µg in 1 µl DMSO) or DMSO was injected (ICV) twice within 24 h for each treatment paradigm. DMI was administered 23.5 h and 1 h before test. DMI significantly decreased immobility (left) and increased climbing behavior (right) but this effect was not seen in the K252a-treated animals (n = 9 rats/group). VNS was administered 3 times over 24 h. VNS significantly increased swimming behavior. However, this effect was not seen in the K252a-treated animals. VNS did not affect climbing in either DMSO- or K252a-treated animals (n for DMSO/Vehicle = 9, DMSO/DMI = 9; K252a/Vehicle = 9; K252a/DMI = 9;

DMSO/Sham = 8; DMSO/VNS = 8; K252a/Sham = 7; K252a/VNS = 9) (B). *Significantly different from relevant control value, $p < 0.05$ (Two-way ANOVA, followed by planned comparisons using Fisher's LSD test).

Figure 6. Immunohistochemical detection of TrkB-Fc (20 μ g in 10 μ l) distribution at 15 min, 3 h and 8 h post-ICV administration (A). Effect of ICV administration of TrkB-Fc on TrkB phosphorylation at Y705 in the dorsal hippocampus induced by acute DMI (10 mg/kg, IP) (B) and VNS (C) treatments

Representative brain sections for each time point are shown. At the 15 min time-point, staining was detected outlining the ventricular spaces in sections anterior and posterior to the site of injection. Staining was also seen in the dorsal region adjacent to the ventricle at site of injection. 3-h post ICV injection, staining was detected bilaterally adjacent to the ventricles in the plane of the injection site, and in the dorsal hippocampus. 8-h post ICV

injection, staining was detected in the lateral and ventral region of hippocampus (A). The ability of acute (2 h) DMI (B) or VNS (C) treatments to induce phosphorylation of TrkB at Y705 was abolished by prior administration of TrkB-Fc (20 μ g in 10 μ l, ICV). PhosphoY705 values are normalized against Total TrkB values (n for PBS/Vehicle = 7, PBS/DMI = 7; TrkB-Fc/Vehicle = 7; TrkB-Fc/DMI = 7; PBS/Sham = 8; PBS/VNS = 9; TrkB-Fc/Sham = 9; TrkB-Fc/VNS = 8) *Significantly different from relevant control, $p < 0.05$ (Two-way ANOVA followed by planned comparisons using Fisher's LSD test). Digital images represent immunoreactive bands for pY705 TrkB that aligns with total TrkB at ~120 -145 kDa, Total TrkB (~120-145 kDa) and beta-Actin (~50 kDa).