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Tricyclic antidepressant treatment evokes regional changes in neurotrophic factors over time within the intact and degenerating nigrostriatal system

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Abstract

In addition to alleviating depression, trophic responses produced by antidepressants may regulate neural plasticity in the diseased brain, providing not only symptomatic benefit but potentially slowing the rate of disease progression in Parkinson's disease (PD). Recent *in vitro* and *in vivo* data provide evidence that neurotrophic factors such as brain derived-neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) may be key mediators of the therapeutic response to antidepressants. As such, we conducted a cross-sectional time-course study to determine whether antidepressant-mediated changes in neurotrophic factors occur in relevant brain regions in response to amitriptyline (AMI) treatment before and after intrastriatal 6-hydroxydopamine (6OHDA). Adult male Wistar rats were divided into seven cohorts and given daily injections (i.p.) of AMI (5mg/kg) or saline throughout the duration of the study. In parallel, various cohorts of intact or parkinsonian animals were sacrificed at specific time points to determine the impact of AMI treatment on trophic factor levels in the intact and degenerating nigrostriatal system. The left and right hemispheres of the substantia nigra, striatum, frontal cortex, piriform cortex, hippocampus and anterior cingulate cortex were dissected and BDNF and

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GDNF levels were measured with ELISA. Results show that chronic AMI treatment elicits effects in multiple brain regions and differentially regulates levels of BDNF and GDNF depending on the region. Additionally, AMI halts the progressive degeneration of dopamine (DA) neurons elicited by an intrastriatal 6-OHDA lesion. Taken together, these results suggest that AMI treatment elicits significant trophic changes important to DA neuron survival within both the intact and degenerating nigrostriatal system.

Keywords

Neuroprotection; antidepressants; amitriptyline; trophic factors; Parkinson's disease

Introduction

Depression is a common co-morbid disorder that affects almost half of all patients with Parkinson's disease (PD), many of which take antidepressants on a daily basis [1]. Although a number of studies have investigated the trophic response to various antidepressant drugs [2-11], most focus on changes within mesolimbic brain structures. Therefore, little is known about whether antidepressant therapy impacts trophic factors within the nigrostriatal dopamine (DA) system. The trophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) are of particular importance for nigrostriatal DA neurons vulnerable to degeneration in PD [12]. While both factors have shown promise for PD pre-clinically [13-24], only GDNF has been investigated for utility in human trials. Multiple open label clinical trials demonstrated significant symptomatic improvement in PD patients that received intraputamenal GDNF infusion [25-27]. However, a randomized, controlled trial of intraputamenal GDNF infusion yielded negative results [28], thereby dampening the enthusiasm for exogenous delivery of this protein in the clinic. Though unexpected, this finding does not negate the positive data supporting the neuroprotective potential of trophic factors; it merely indicates that *exogenous* delivery of trophic support may not be an optimal strategy, especially in end stage PD. Therefore, treatments that induce the synthesis or release of *endogenous* factors in the brain, particularly earlier in the disease process, may provide a more effective strategy for treating PD patients [29, 30].

Psychopharmacological agents such as antidepressants have been shown to modulate important signaling pathways involved in cell survival and plasticity [3-5], thereby suggesting they may have more pleiotrophic ability than previously anticipated. Such studies provide evidence that antidepressant treatment could evoke changes that induce neuroprotection in neurodegenerative diseases, including PD. The trophic response elicited by chronic antidepressant treatment has been well documented within mesolimbic regions involved in depression and mood disorders; however, little is known about whether similar changes occur in the nigrostriatal pathway and hence may provide dual therapeutic value for PD patients. Additionally, there are conflicting data concerning the onset and duration of antidepressant-mediated trophic responses [31, 32]. Therefore, we performed a cross-sectional time-course study to determine the effects of chronic antidepressant treatment on BDNF and GDNF levels in brain regions relevant to PD. As tricyclic antidepressants

(TCAs) have been shown to be more efficacious in treating depression in PD patients [33, 34], we opted to examine the specific TCA amitriptyline (AMI), which is often prescribed to patients with PD [35]. Furthermore, we recently reported that AMI, but not other antidepressants, were associated with a delay in the need to start dopaminergic therapy in an early cohort of patients with PD [36], suggesting it may have disease-modifying properties. More recently, our laboratory demonstrated that the low dose of AMI used in this study afforded significant neuroprotection to the nigrostriatal system in a rat toxin model of parkinsonism [37]. We report here that chronic AMI treatment differentially regulates BDNF and GDNF in the intact and degenerating nigrostriatal system, and that AMI significantly retards the progression of nigral dopamine neuron loss.

Methods

Animals

Animals were housed and treated following the National Institutes of Health guidelines [38]. Each experiment utilized adult (4-6 month old) male Wistar rats (200-225g) as subjects. Rats were obtained from Harlan Animal Research Laboratory (Indianapolis, IN). The animal facility at the University of Cincinnati is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and complied with all Federal animal care and use guidelines. The Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati, where these studies were performed, approved all protocols pertaining to these experiments.

Experimental Design

Rats were acclimated to their new environment for 24 hours before beginning experiments then randomized and divided into four groups (saline-no lesion, amitriptyline (AMI)-no lesion, saline +lesion, AMI +lesion) and given daily intraperitoneal (i.p.) injections of sterile saline (Henry Schein) or 5mg/kg of amitriptyline hydrochloride (AMI, Sigma) for 14 days (Fig. 1A). This 14-day pretreatment regimen and low dosage was based on previous studies indicating that this timing is important to achieve antidepressant efficacy [3, 5, 11]. On day 14, rats in the appropriate groups (+lesion) received unilateral intrastratial injection of the DA neurotoxin 6-hydroxydopamine (6-OHDA), after which all rats continued to receive daily i.p. AMI or saline injections for the remainder of the experiment (four weeks). Lesioned and non-lesioned cohorts of rats from both saline and AMI groups were sacrificed on days 14, 17, 24 and 42, which corresponds to the following days post-lesion: day 0 (D0), day 3 (D3), day 10 (D10) and day 30 (D30). All rats were sacrificed at prescribed time-points and brains extracted and processed for either immunohistochemical or ELISA analyses.

To assess trophic factor levels within the nigrostriatal system, we examined BDNF and GDNF protein in the SN and striatal structures. However, BDNF is not produced in the striatum [39, 40], yet receives BDNF protein via anterograde/retrograde transport from various interconnected regions including the SN [41], hippocampus [42, 43], frontal [39, 40, 44], piriform [45, 46] and anterior cingulate cortices [47-50] (Fig. 1B). Therefore, we

extended the scope of the project to include these structures, and each hemisphere was microdissected and processed as an independent sample as described previously [51].

6-OHDA Lesions

Prior to surgery, all rats were anesthetized (30 mg/kg, pentobarbital, i.p.) and placed into a stereotaxic device. The 6-OHDA (dissolved in a 0.2% ascorbic acid-physiological saline) was made immediately prior to surgery and kept on ice. All rats received 2µl of 6-OHDA (5µg/µl) into the ipsilateral left striatum (AP +0.7, ML +3.0, DV -5.5 from skull) at a rate of 0.5µl per minute. After each injection, the needle was left in place for 2 minutes and then slowly withdrawn. Lesions were verified by stereological assessment of tyrosine hydroxylase immunoreactive (THir) neurons within the substantia nigra pars compacta (SNpc).

Immunohistochemistry

Animals were euthanized via pentobarbital overdose (60mg/kg) and intracardially perfused with 0.9% saline followed by ice cold 4% paraformaldehyde (PFA) in 0.1 M PO⁴ buffer. Brains were extracted and post-fixed in 4% PFA for 24 hours and sunk in 30% sucrose. Brains were cut into 40µm thick sections on a freezing microtome. A 1:6 series of coronal sections was double labeled for tyrosine hydroxylase (TH) (marker of dopamine neurons) and NeuN (general neuronal marker) using the free-floating method. A sequential approach was utilized to visualize these proteins using antibodies from the same host (mouse)[52]. Tissue was incubated in 0.3% H₂O₂ for 45 minutes, rinsed and blocked in 10% normal goat serum (1 hour) then incubated in primary mouse anti-NeuN antibody (1:500; Chemicon) overnight at 4° C. Following primary incubation, sections were incubated in biotinylated secondary antisera against mouse IgG (1:400, Chemicon) followed by the Vector ABC detection kit employing horseradishperoxidase (Vector Labs). Antibody labeling was visualized by exposure to 0.5 mg/ml 3,3' diaminobenzidine (DAB), 2.5mg/ml nickel ammonium sulfate and 0.03% H₂O₂ in Tris buffer. Tissue was rinsed and blocked again overnight at 4°C. The following day, tissue was incubated in mouse anti-TH antibody (1:4000, Chemicon) for four hours, followed by biotinylated secondary antisera against mouse IgG (same as above) and the Vector ABC detection kit. Labeling was visualized using the NovaRed kit (Vector Labs) and sections were mounted on subbed slides, dehydrated to xylene and coverslipped with Cytoseal (Richard-Allan Scientific). Since TH is localized to the cytoplasm, any cross reactivity to the biotinylated secondary antisera against mouse IgG labeling did not interfere with nuclear NeuN visualization.

ELISA

Animals were anesthetized (60mg/kg, pentobarbital, i.p.) and perfused with heparinized saline (0.9%). Brains were flash frozen in 3-methyl butane (Fisher) then stored at -80°C. 1-2 mm coronal slabs were blocked utilizing a brain blocker (Zivic, Pittsburg, PA) and both hemispheres of the SN, striatum, hippocampus, frontal cortex, anterior cingulate cortex (ACC) and the piriform cortex were microdissected at a constant - 12°C on a cold plate (Teca, Chicago,IL) and stored at - 80°C until analysis. Samples were homogenized on ice in 200ul of lysis buffer (M-PER; Pierce) with protease inhibitors (Sigma-Aldrich). The Pierce BCA Protein Kit (Rockford, IL) was utilized for protein determination. The homogenate

was centrifuged at 4°C for 20 minutes at 14,000g and the supernatant was collected. ELISAs were run using the Immunolon 4 HBX 96 well plate according to manufacturer's instructions (GDNF/BDNF Promega Emax ImmunoAssay Kit). Samples were read (<30 minutes) on a spectrophotometer (Multiskan Spectrum, Thermolab Systems) with unknown values interpolated against standard curves.

Stereology for Cell Counts

MicroBrightfield stereological software (MBF Bioscience, Inc.) was used for stereological quantitation of TH and NeuN cells. Outline of substantia nigra pars compacta (SNpc) region of interest (ROI) was performed under low magnification (1.25X) and 20% of the designated area was sampled via a random series of counting frames systematically distributed throughout the contour. Cell counts were performed using the optical fractionator probe under high magnification (60X with oil) by an investigator blind to the animal experimental status. For the ROI, a z-stack of images was acquired at each sampling site within the stereological probe. Cells were counted by focusing through the tissue at 1-2 µm intervals (dissector height was 25µm with top/bottom guard zones of 2.5µm), and then a marker was placed over each NeuN/THir or NeuNir/TH negative neurons within the counting frame (50x50µm). Between 50 and 300 objects were counted to generate the stereological estimates. The total number of stained neurons (N) in the regions of interest was calculated using the following formula: $N = N_V \cdot V_{ROI}$ where N_V is the numerical density and V_{ROI} is the volume of the region of interest. The volume was calculated according to the procedure of Cavalieri and variability within groups was assessed via the Coefficient of Error (CE). The Gunderson method was used for calculating the CE value (< 0.1) for the population estimate [53].

Statistical Analysis

AMI-mediated differences in trophic factor levels over time in intact animals were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-hoc analysis comparing all time points to the baseline control value. Differences between groups in 6-OHDA lesioned animals over time were analyzed by two-way ANOVA. Comparisons in THir and NeuNir/TH negative neurons between groups were analyzed using a one-way ANOVA. When appropriate, post-hoc comparisons were made between groups using the Student-Newman-Keuls or Bonferonni method. The level of significance was set at $P = 0.05$.

Results

Regional Distribution of BDNF and GDNF in Rat Brain

Baseline values of BDNF and GDNF measured in different brain structures are depicted in Table 1. BDNF and GDNF levels varied significantly across regions, but were in line with previous reports of BDNF and GDNF expression in brain [54, [54-56].

Effects of Amitriptyline (AMI) on BDNF and GDNF Levels in the Intact Brain

BDNF—ELISA results demonstrated that hippocampal BDNF was significantly increased compared to baseline levels and remained elevated after 24 days of AMI treatment ($F(4,$

62)=5.217, $p<0.001$; Fig. 2, A). Previous studies indicate that hippocampal BDNF is increased in response to antidepressant treatment [54]; therefore, this structure served as a positive control for AMI efficacy. In the SN, BDNF was significantly increased after 24 days of AMI treatment and remained elevated for the duration of the study ($F(4, 59)=3.764$, $p=0.0009$; Fig. 2B). In the striatum, BDNF was significantly decreased after 24 days of AMI treatment ($F(4, 52)=8.472$, $p<0.001$; Fig. 2C). In the frontal cortex, AMI did not elicit any significant changes in BDNF ($p > 0.05$; Fig. 2D). In the ACC, BDNF was significantly decreased after 24 days of AMI treatment ($F(4, 58)=4.627$, $p=0.003$; Fig. 2E). In the piriform cortex, there was a transient but significant increase in BDNF after 14 days of AMI treatment, but then levels significantly decreased after 24 days ($F(4, 60)=16.22$, $p<0.0001$; Fig. 2F).

BDNF—A transient, but significant increase in hippocampal BDNF was observed after 14 days of AMI treatment ($F(4, 49)=5.257$, $p=0.002$; Fig. 3A). In line with BDNF results, SN BDNF levels were significantly increased after 24 days of treatment with AMI and remained elevated ($F(4, 52)=3.740$, $p=0.0095$; Fig. 3B). Striatal BDNF did not change in response to chronic AMI treatment ($p>0.05$; Fig. 3, C). Similarly, AMI did not elicit any significant changes in BDNF in the frontal cortex (Fig. 3D). In contrast to BDNF results, ACC BDNF was significantly increased at 14 days (compared to baseline levels) as a result of AMI treatment ($F(4, 50)=10.38$, $p<0.001$; Fig. 3, E), then returned to baseline over time. BDNF levels were not altered in the piriform cortex in response to chronic AMI treatment ($p>0.05$; Fig. 3, F). Combined, these results suggest AMI mediates substantial trophic changes over time within the intact nigrostriatal system that may significantly impact DA neuron survival.

DA Degeneration Post 6-OHDA Lesion

Population estimates were derived for both TH immunoreactive (ir) and NeuNir neurons throughout the lesioned substantia nigra in saline- and AMI-treated rats. There were no significant differences in the total number of THir cells in the intact hemisphere between groups ($F(1, 31) = 2.78$; $p>0.05$) or over time ($F(3, 31) = 0.69$; $p>0.05$) and the combined average population estimate was $15,683.00 \pm 819.89$. Additionally, there were no significant differences between groups ($F(1, 32) = 1.90$; $p>0.05$) or over time ($F(3, 32) = 0.14$; $p>0.05$) for total NeuNir counts on the intact side and the combined average population estimate was $30,603.00 \pm 1403.30$. Stereological assessment showed that in the SNpc ipsilateral to intrastriatal 6-OHDA injection THir neurons decreased by approximately 25% in both treatment groups 10 days postlesion. Neurodegeneration continued in the saline-treated group (-60%), whereas it was halted in the AMI-treated group (-32%) 30 days post-lesion. A two-way ANOVA revealed a significant overall effect for time ($F(3, 33)=11.89$, $p<0.0001$). *Post hoc* analyses indicate significant differences between groups at the 30-day time point with AMI treatment associated with significantly higher numbers of THir neurons (Fig. 4A; $p<0.05$). To confirm that the extent of THir neuron loss was the result of neuron death and not phenotype down-regulation, THir neuron counts were compared to total NeuNir neuron counts. Results show an overall effect for time demonstrating that the number of neurons declined significantly over time ($F(3, 55)= 8.48$, $p<0.0001$; Fig. 4B). In saline-treated rats, the amount of THir neuron loss was greater at day 3 (-2224.51 ± 1982.95) and day 10 (-4163.29 ± 2163.38) compared to the total NeuNir neuron loss at day 3 (-587.19 ± 2712) and

day 10 (-1623.79 ± 1584.43) post-lesion. However, by day 30 post-lesion, THir neuron loss (-9815.89 ± 1724.57) was comparable to total NeuNir neuron loss (-8961.97 ± 1482.58). Similarly, in AMI-treated rats, the amount of THir neuron loss was greater at the early time points (day 3: -1171.65 ± 952.84 and day 10: -5706.63 ± 1819.03) relative to the total NeuNir neuron loss at the early time points (day 3: -981.95 ± 933.19 and day 10: -2766.99 ± 1884.75) post-lesion. Yet, at the 30-day time point post-lesion, THir neuron loss (-4138.34 ± 1040.97) was equivalent to total NeuNir neuron loss (-4346.23 ± 1264.04). *Post hoc* analysis revealed a significant difference between the number of THir neurons in AMI- and saline-treated rats 30 days post-lesion (-4138.34 ± 1040.97 vs. -9815.89 ± 1724.57 ; $p < 0.05$). Combined, these data show that for both AMI- and saline-treated rats, loss of THir (DA phenotype) precedes overt neuron loss at the early time points post-lesion. However, after 30 days post-lesion, both populations decline in comparable numbers indicating that a loss of TH phenotype reflects actual neuron loss. Additionally, AMI treatment significantly attenuated the magnitude of nigral DA neuron loss 30 days post-lesion.

Effects of Amitriptyline (AMI) on BDNF and GDNF Levels in Brain During Nigrostriatal Degeneration

BDNF—In the 6-OHDA lesioned brain, there was a significant interaction between treatment and time for hippocampal BDNF ($F(2, 36)=11.37$, $p < 0.0001$), suggesting that AMI impacted BDNF levels over time. *Post hoc* analysis revealed that hippocampal BDNF was significantly increased in AMI treated animals at the 30-day time point compared to either treatment at other time points (Fig 5A). Furthermore, this increase was significantly elevated compared to saline treated animals at the 30-day time point (~ 2.5 fold; $p < 0.0001$), suggesting there is a temporal increase in hippocampal BDNF mediated by AMI that occurs independent of the lesion. In the SN, there was a significant effect of treatment ($F(1, 25)=5.550$, $p < 0.05$) suggesting that AMI impacted BDNF levels over time. At the 3 day post-lesion time point, *post hoc* analysis indicates SN BDNF was significantly elevated in response to AMI (~ 2.6 fold; $p < 0.05$) compared to saline (Fig 5B); but that levels normalized by the 30 day time-point. In the lesioned striatum, there was no significant effect overall; however, there was a substantial (-56%), albeit non-significant, AMI-mediated decrease in BDNF levels over time (Fig. 5C). In the frontal cortex (Fig. 5D), ACC (Fig. 5E) and piriform (Fig. 5F) cortices, there was no effect of time or treatment ($p > 0.05$); however, there was a trend suggesting AMI-mediated a decrease in ACC BDNF over time ($p = 0.06$).

GDNF—In contrast to BDNF, there was no significant effect of time or treatment for GDNF in the hippocampus ($p > 0.05$; Fig. 6A). However, there was a significant effect of treatment on GDNF levels in the SN ($F(1, 29)=4.37$, $p < 0.05$; Fig 6B). *Post hoc* analysis reveals a significant increase in SN GDNF at the early time point immediately post lesion compared to saline control ($p < 0.05$), which returns to control levels by the 10-day time-point. There were no significant effects for striatal ($p > 0.05$, Fig. 6C) or frontal cortex ($p > 0.05$; Fig. 6D) GDNF levels. There was an overall effect of treatment for ACC GDNF levels ($F(1, 24)=5.32$, $p < 0.05$). *Post hoc* analyses indicate that AMI-mediated a significant increase in ACC GDNF at the early time points compared to saline treated animals post-lesion, and these levels returned to normal by the 10-day time-point (Fig. 6E). There were no significant effects of time or treatment for GDNF levels in the piriform cortex ($p > 0.05$; Fig 6F). Taken

together, results illustrate a divergent pattern of trophic changes elicited by antidepressant treatment within the degenerating brain.

Discussion

The findings of this study are highly relevant to people afflicted with PD because these individuals often experience depression and take antidepressants daily. In our evaluation of the temporal response of trophic support in the nigrostriatal system, the current study: 1) extends the scope of our previous finding that AMI treatment is indeed neuroprotective [37], and 2) shows that chronic AMI treatment elicits a trophic response that is both dynamic and divergent depending on the region examined. Our data demonstrate that within the intact nigrostriatal system, AMI mediates a more pronounced effect on BDNF than GDNF. Pertinent to the notion that AMI may provide ongoing neuroprotection for PD patients, we observed a temporal increase in BDNF levels within both the SN and HIPP, which was maintained with chronic drug administration. Conversely, levels of BDNF were reduced by half over the course of treatment in the striatum and interconnected regions (ie., ACC and piriform cortices). Of potential added trophic benefit, AMI also increased GDNF in the SN in a time-dependent manner, which again was maintained through the extent of drug treatment. The return of GDNF to basal levels over time in the HIPP and ACC contrasts with that seen in the SN, and its significance for antidepressant activity is uncertain. While it is reasonable to suggest that the increased trophic support elicited by AMI is a likely contributor to the nigral DA neuron protection observed in this and our previous study, it is unclear why BDNF levels are decreased in the striatum, anterior cingulate and piriform cortices, and what impact this might have on the dopamine circuitry or the survival of neuronal sub-types that reside therein.

Although it is important to understand how antidepressant treatment impacts the intact nigrostriatal system, it is essential to detail these effects in the degenerating system. To determine the effect of AMI on nigral DA degeneration, the extent of DA neuron loss was measured at select time points after 6-OHDA lesion. While AMI binds primarily to norepinephrine and serotonin transporters and its affinity for the dopamine transporter is negligible [57], animals did not receive an injection of AMI/saline on the day of surgery to ensure AMI did not impede the uptake of 6-OHDA. Since AMI is rapidly cleared from plasma and brain (<12 hours) [58], we feel confident that AMI was not present to interfere with the uptake of 6-OHDA. Furthermore, we've previously shown that chronic AMI pre-treatment does not alter DAT levels [37], suggesting 6-OHDA could be readily taken up by dopamine neurons to induce a lesion. Results indicate both AMI- and saline-treated rats lost equivalent numbers of SN DA neurons at early post-lesion time points (ie., 3-10 days post-lesion); however, neurodegeneration continued in the saline treated animals, whereas it was halted in the AMI-treated group. We also report a dynamic trophic response mediated by AMI in the SN immediately post-lesion. This robust increase in BDNF and GDNF soon after the insult importantly suggests that AMI-mediated trophic support of DA neurons, may be a means to preserve these cells during the axonopathic "die back" that has been shown to occur early in the disease [59]. This confirms our previous result highlighting the neuroprotective ability of AMI [37] and is in line with previous reports showing antidepressants from multiple classes elicit neuroprotective effects in neurodegenerative

diseases [33, 60, 61]. For example, nortriptyline (the metabolite of AMI) has been shown to prevent the death of motor neurons in an amyotrophic lateral sclerosis (ALS) mouse model and prevent cell death in a Huntington's disease (HD) cellular model. These effects were attributed to nortriptyline's anti-apoptotic effects (i.e., inhibition of release of cytochrome c and activation of caspase 3). Additionally, the SSRI sertraline has been shown to prolong survival, improve motor performance and decrease brain atrophy in the R6/2 Huntington's disease (HD) mouse model [61]. The beneficial effects of sertraline were attributed to its ability to increase hippocampal BDNF and enhance neurogenesis. Similarly, treatment with the SSRI, paroxetine, prevented degeneration of nigrostriatal DA neurons, increased striatal DA levels, and improved motor function in a mouse model of PD [33]. These authors attribute paroxetine's neuroprotective effects to anti-inflammatory mechanisms and reduction of oxidative stress. Finally, a more recent study reports that the SSRI fluoxetine ameliorates motor deficits in a transgenic model of multiple systems atrophy (MSA), with a concomitant decrease in neurodegenerative pathology in the basal ganglia, neocortex and hippocampus [62]. These investigators [62] attribute the neuroprotective effects mediated by antidepressant treatment to increased levels of hippocampal BDNF and GDNF, as well as ERK activation. Taken together, existing data suggests that antidepressants may provide neuroprotection to DA neurons via a number of different mechanisms and may have dual therapeutic value in treating both the motor and non-motor features of PD.

As previously reported, some of the therapeutic effects elicited by antidepressants may be due to increased trophic factor levels in the brain. Of all the neurotrophic factors, BDNF [13-17, 63] and GDNF [18-24, 64-72] have shown the most promising results in animal models of PD. These factors exert their effects by binding to tyrosine kinase receptors (TrkB and GDNF α /RET, respectively) that activate tyrosine kinases through receptor dimerization, followed by autophosphorylation and resultant intracellular signaling. The intracellular pathways involved in cell survival include the extracellular signal-regulated-protein kinase/mitogen-activated protein kinase (ERK/MAPK) [7, 73] and phosphatidylinositol-3-kinase (PI3K) signaling pathways [74-76]. Moreover, the expression of these factors is activity dependent and is involved in neuronal adaptation and plasticity. Although antidepressant-mediated changes have been demonstrated in brain regions involved in depression, little is known about the morphological and biochemical changes induced by antidepressants within the degenerating nigrostriatal system. Therefore, we examined levels of BDNF and GDNF at various time points after lesion in AMI- and saline-treated rats. This cross-sectional time course study allowed us to visualize the amount of DA neuron loss relative to the changes in BDNF or GDNF at specific time-points after lesion.

The present study demonstrates AMI treatment was associated with a significant increase in SN BDNF early post-lesion, suggesting it may participate in the nigrostriatal neuroprotection afforded by AMI treatment. Additionally, levels of GDNF were increased immediately post-lesion in both the SN and ACC, which may further contribute to the AMI-mediated preservation of the DA system. Hippocampal BDNF was significantly elevated over time by AMI treatment, suggesting antidepressant efficacy despite ongoing degeneration. In fact, we previously demonstrated that the low dose and time-course of AMI administration utilized in this study exhibited antidepressant activity and attenuated motor deficits induced by the 6-OHDA lesion [37]. Although not specifically investigated, the

AMI-mediated increase in hippocampal BDNF may also preserve the loss of hippocampal neurogenesis associated with PD-related depression [77]. Taken together, the present findings indicate the AMI-mediated trophic response elicited here could potentially restore diminished trophic factor levels in the aged or injured brain, thereby providing dual therapeutic value by attenuating both depression and neurodegeneration.

In contrast to the upregulation of BDNF in the SN and HIPPO, striatal BDNF decreased substantially post-lesion. Although striatal BDNF levels are equivalent in AMI- and saline-treated lesioned rats at the early time-points, over time BDNF levels decline only in AMI-treated rats. Contrary to previous reports utilizing near-complete lesions that show elevated BDNF in the striatum post-lesion [78, 79], the present findings indicate striatal BDNF levels do not change substantially in response to a partial lesion. However, it is important to note that previous reports used different lesion parameters and also compared the lesioned hemisphere to the contralateral (non-lesioned) hemisphere, which we have found is changed post-lesion (data not shown). Accordingly, prior reported changes were not normalized to baseline levels in lesioned animals. Interestingly, after 6 weeks of AMI treatment, striatal BDNF levels fall to half (~50%) of control levels in both the intact and lesioned animals. This provides strong evidence that such an effect is the direct result of chronic AMI treatment, independent of ongoing neurodegeneration. Additionally, it shows that antidepressants can elicit divergent effects across the brain. To this end, BDNF has been shown to have very different effects in animal models of depression depending on the brain region involved. For example, BDNF is best characterized in the hippocampus where its induction by antidepressants is important for alleviating depressive symptoms [5, 80]. Conversely, local depletion of BDNF in the ventral tegmental area (VTA)/nucleus accumbens pathway also elicits an antidepressant-like effect [81]. Furthermore, intact BDNF function in the VTA is required for the development of social aversion in mice [82, 83]. These results are in opposition to previous reports linking increased BDNF to antidepressant efficacy. However, the increase in BDNF relative to antidepressant action has been shown primarily in the hippocampus. It is not clear why striatal BDNF decreases in response to chronic AMI treatment. Furthermore, it is not known whether this effect occurs with other antidepressants and what impact the striatal BDNF deficiency may have on the DA system or the neuronal subtypes that rely on BDNF for survival. This will be of interest in future studies.

In summary, our results show chronic AMI treatment elicits effects in brain regions that extend beyond the mesolimbic system and that AMI differentially regulates levels of BDNF and GDNF depending on the structure, and time of exposure to drug. The current studies also serve to confirm our previous report that AMI has neuroprotective effects and can halt the continuing degeneration of DA neurons elicited by intrastratial 6-OHDA. Furthermore, our findings suggest that the most pronounced BDNF and GDNF increases are associated with the DA neuron cell bodies in SN, rather than DA terminals in striatum. Thus, based on the correlative relationship documented here between AMI, BDNF/GDNF, and DA neuron survival, it is reasonable to suggest that chronic AMI treatment induces trophic changes that may help retard neurodegeneration in the diseased brain. These data provide rationale for continuing investigations to 1) confirm a causal relationship between AMI effects on these trophic factors and DA neuron survival, 2) determine whether these factors work

independently or synergistically, and 3) delve into additional factors which might be activated by AMI for development as targetable therapeutics in PD.

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Highlights

Chronic amitriptyline treatment significantly increases BDNF and GDNF in substantia nigra

Chronic amitriptyline treatment substantially decreases BDNF in striatum

Chronic amitriptyline treatment attenuates dopamine neuron loss

Tricyclic antidepressants may have dual therapeutic value in Parkinson's disease

Figure 1. Experimental design and nigrostriatal interconnectivity

The experimental timeline depicts the cross-sectional time course design highlighting specific time-points at which select cohorts are treated and sacrificed (A). Time course in gray (Days 0-42) shows sacrifice times for non-lesioned animals treated with saline or AMI. Structures dissected at these times are indicative of the trophic response elicited by saline or AMI treatment in intact animals. Time course in black (Days 0-30) depicts sacrifice times post-lesion for animals treated with saline or AMI. Structures dissected at these times are indicative of the trophic response elicited by saline or AMI in the degenerating nigrostriatal system. Representative map detailing the interconnectivity between dissected structures of interest (B). *Abbreviations:* AMI=amitriptyline hydrochloride, D=day, Sac=sacrifice, 6OHDA=6-hydroxydopamine, IHC=immunohistochemistry, ELISA=enzyme-linked immunosorbent assay, STR=striatum, HIPP=hippocampus, SN=substantia nigra, FCX=frontal cortex, ACC=anterior cingulate cortex, PIR=piriform cortex

Figure 2. Temporal regulation of BDNF in response to AMI

Adult rats received saline or AMI (5mg/kg, i.p.) for 14, 17, 24 or 42 days. Each hemisphere of the region of interest was dissected on ice and analyzed independently with ELISA for BDNF. Levels of BDNF are depicted for each of the following regions: Hippocampus (A), substantia nigra (B), striatum (C), frontal cortex (D), anterior cingulate (E) and piriform cortex (F). Data from AMI-treated rats are depicted in grey and data from saline-treated animals are depicted in black. Values are expressed as a percentage of absolute values in the saline control group. Levels of significance determined by Dunnett's multiple comparison post-hoc analysis comparing all time points to the baseline control value. Data represent mean $\pm$ SEM and are combined from two separate experiments. Abbreviations: AMI: amitriptyline, TXT: treatment, BDNF: brain derived neurotrophic factor * $p < 0.05$, ** $p < 0.01$

Figure 3. Temporal regulation of GDNF in response to AMI

Adult rats received saline or AMI (5mg/kg, i.p.) for 14, 17, 24 or 42 days. Each hemisphere of the region of interest was dissected on ice and analyzed independently with ELISA for GDNF. Levels of GDNF are depicted for each of the following regions: Hippocampus (A), substantia nigra (B), striatum (C), frontal cortex (D), anterior cingulate (E) and piriform cortex (F). Data from AMI-treated rats are depicted in grey and data from saline-treated animals are depicted in black. Levels of significance determined by Dunnett's multiple comparison post-hoc analysis comparing all time points to the baseline control value. Values are expressed as a percentage of absolute values in the saline control group. Data represent mean $\pm$ SEM and are combined from two separate experiments. Abbreviations: AMI: amitriptyline, TXT: treatment, GDNF: glial-derived neurotrophic factor * $p < 0.05$, ** $p < 0.01$

Figure 4. Amitriptyline slows the progressive loss of dopamine (DA) neurons in the 6- OHDA rat model of PD

Stereological population estimates show DA neuron loss is significantly attenuated in the AMI-treated animals compared to the saline-treated animals 30 days post-lesion (A). To confirm that the extent of DA (THir) neuron loss was the result of cell death and not phenotype downregulation, THir cell counts were compared to NeuN counts. Results indicate both populations of cells decline in comparable numbers by the 30-day time point (B); however, there is significantly less THir cell loss in the AMI group compared to the saline group. For comparison, dashed line illustrates percent lesion for control animals at 30-day time point. Data represent mean $\pm$ SEM and are combined from two separate experiments. Abbreviations: AMI: amitriptyline, TH: Tyrosine hydroxylase, THir: tyrosine hydroxylase immunoreactive, NeuN: neuronal nuclei, NeuNir: NeuN immunoreactive. Panel A: * $p < 0.05$ compared to saline D0, \$ $p < 0.05$ compared to AMI D0, ## $p < 0.01$ compared to saline D3, ^ $p < 0.01$ compared to AMI D3; Panel B: * $p < 0.05$ compared to Saline TH at D30

Figure 5. BDNF response to amitriptyline (AMI) post-lesion

BDNF is differentially regulated in response to chronic antidepressant treatment and/or lesion. The region of interest was dissected and each hemisphere was analyzed independently with ELISA for BDNF. Adult rats were treated with saline or AMI (5mg/kg, i.p.) for 2 weeks prior to receiving 6-OHDA lesions on day 14. Subsets of these rats were sacrificed at 3, 10 or 30 days post-lesion. Levels of BDNF are depicted for each of the following regions: Hippocampus (A), substantia nigra (B), striatum (C), frontal cortex (D), anterior cingulate (E) and piriform cortex (F). Values are expressed as a percentage of saline lesioned controls. Data represent the mean $\pm$ SEM and are combined from two separate experiments. ** p <0.01, **** p <0.0001, \$\$\$ p <0.001

Figure 6. GDNF response to amitriptyline (AMI) post-lesion

GDNF is differentially regulated in response to chronic antidepressant treatment and/or lesion. The region of interest was dissected and each hemisphere was analyzed independently with ELISA for GDNF. Adult rats were treated with saline or AMI (5mg/kg, i.p.) for 2 weeks prior to receiving 6-OHDA lesions on day 14. Subsets of these rats were sacrificed at 3, 10 or 30 days post-lesion. Levels of GDNF are depicted for each of the following regions: Hippocampus (A), substantia nigra (B), striatum (C), frontal cortex (D), anterior cingulate (E) and piriform cortex (F). Values are expressed as a percentage of saline lesioned controls. Data represent the mean $\pm$ SEM and are combined from two separate experiments. * $p < 0.05$, ** $p < 0.001$

Table 1

Baseline BDNF and GDNF levels in saline-treated rats

Regional Levels of BDNF and GDNF in Rat Brain		
BDNF		
Region	N	Levels pg/mg protein
HIPP	13	37.99 ± 3.95
SN	8	46.28 ± 1.95
STR	8	30.20 ± 3.15
FCX	11	53.42 ± 6.73
ACC	13	30.95 ± 5.68
PIR	13	58.96 ± 3.28
GDNF		
Region	N	Levels pg/mg protein
HIPP	8	79.54 ± 24.58
SN	12	245.3 ± 53.09
STR	14	176.3 ± 32.98
FCX	12	220.3 ± 27.81
ACC	6	183.3 ± 87.08
PIR	14	139.0 ± 35.79

Values represent baseline BDNF or GDNF levels (mean ± SEM) obtained from intact rats treated with saline for two weeks. Data are combined from two different experimental control groups. Each hemisphere of the region of interest (ROI) was processed independently and represents one sample. Variability in sample size is due to loss or insufficient recovery of protein.