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Effects of nerve growth factor (NGF), fluoxetine, and amitriptyline on gene expression profiles in rat brain

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ABSTRACT

Evidence suggests that nerve growth factor (NGF) may have antidepressant properties but the pharmacological mechanisms remain unknown. Previously, we found that NGF improved performance in the forced swim test in Flinders Sensitive Line rats, but did not appear to have similar biochemical actions with the antidepressant fluoxetine. Gene expression profiles for neurotransmitter receptors and regulator-related genes in the amygdala/hippocampus were determined in rats treated for 14 days with NGF, fluoxetine, amitriptyline, or saline. Gene expression was measured using an RT^2 profiler PCR Array System to determine the basis for this effect. Compared with saline, there were numerous genes with significantly altered mRNA levels in the amygdala/hippocampal region. Overlap was found between the mRNA levels of genes altered by NGF and the two antidepressant medications including genes related to the cholinergic and dopaminergic systems. However, decreased mRNA levels of Drd5, Sstr3, Htr3a, and Cckar genes in the amygdala/hippocampus were uniquely regulated by NGF. The results of this study are consistent with a previous conclusion that the antidepressant effects of NGF are mediated through non-traditional receptors for traditionally considered neurotransmitters and may suggest a particular utility of NGF in treating comorbid depression and addiction.

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1. Introduction

Unipolar depression has been identified as one of the largest public health problems worldwide, accounting for almost 5% of the global health burden (Chisholm et al., 2004). The past half century has seen a number of antidepressant medications become common treatment for this disorder. These medications typically increase synaptic serotonin and norepinephrine and the traditional view (i.e., the 'monoamine hypothesis of depression') of their efficacy has been through their ability to alter these monoamine neurotransmitter levels. Despite the widespread use of these existing medications, investigation of novel antidepressant treatments are warranted as meta-analyses of antidepressant medication efficacy suggest only 14–18% improvement over placebo (Moncrieff and Kirsch, 2005).

There is growing interest in neurotrophic compounds as potential antidepressant treatments. The most frequently studied neurotrophin in the context of depression is Brain Derived Neurotrophic Factor (BDNF). The 'neurotrophic hypothesis of depression' is supported by stress-induced decreases in hippocampal BDNF and the evidence that antidepressant medications increase hippocampal BDNF (Dwivedi, 2009). Critical review of the literature suggests that BDNF dysfunction may not be sufficient to cause depression, but that BDNF may be an important mediator of the effects of antidepressant medications possibly through its synaptic plasticity actions on hippocampal long term potentiation (LTP) and long term depression (LTD) (Martinowich et al., 2007).

Despite the field's primary focus on BDNF, other neurotrophins may also have efficacy as antidepressant treatments. One emerging candidate is nerve growth factor (NGF); however, to date NGF has been investigated primarily as an Alzheimer's treatment due to its neuroprotective effects on cholinergic neurons that are critical to the development of Alzheimer's disease (Covaceuszach et al., 2009; Tuszynski et al., 2005). Preclinical models of depression suggest that NGF may have antidepressant effects (i.e., use of the forced swim test in Overstreet et al., 2010) although not all studies come to this conclusion (Shirayama et al., 2002). Additional evidence suggests that antidepressant effects seen after NGF administration are realized through an alternative neurobiological

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pathway as compared with established antidepressants. This evidence includes: a lack of differential c-fos expression for either chronic or acute NGF treatment, desipramine-induced blunting of the corticosterone response in fluoxetine – but not NGF-treated rats, and a lack of blunted serotonergic responses (indexed by headshakes following DOI treatment and hypothermia following 8-OH-DPAT treatment) compared with fluoxetine-treated rats (Overstreet et al., 2010). Another intriguing study suggests that intranasally administered NGF may have antidepressant properties in preclinical models as evidenced by behavior in the forced swim test, tail suspension test, and after unpredictable chronic mild stress. Additional evidence for antidepressant properties in this study included increased monoamine levels and c-fos changes in the hippocampus (Shi et al., 2010). Given that the pharmacological actions of existing antidepressants may in fact be most efficacious in severely depressed patients (Fournier et al., 2010) it is important to identify novel antidepressant medications with unique pharmacologies that might have utility in treating a wider range of depression severity.

Considering the promising antidepressant effects of NGF and presumably its novel biochemical actions, a broad-based screen of gene products related to neurotransmitter biosynthesis, uptake, transport, and signaling to understand NGFs actions is warranted. Accordingly, a pathway focused gene expression profiling system (Rat Neurotransmitter Receptors and Regulators RT^2 Profiler™ PCR Array; SABiosciences, Qiagen, Frederick, MD) that surveys 84 genes involved in these processes was utilized to examine differential gene expression in the amygdala/hippocampus of juvenile rats treated for 14 days with NGF, fluoxetine, amitriptyline, or saline as juvenile rats are known to exhibit antidepressant responses to selective serotonin reuptake inhibitors (Reed et al. 2008, 2009).

2. Method

2.1. Animals and drugs

Sprague Dawley rats weighing 100–120 g were purchased from Harlan Laboratories. The animals were housed individually in ventilated cages at a constant temperature of 23.5 ± 2 °C, humidity of 30–70%, and a 12 h light cycle. Water and food were provided *ad libitum*. The rats were divided into four treatment groups of the same average weight. Each group of five rats received once daily treatments by subcutaneous injection of 0.2 ml of 0.995% saline containing 64 ng/kg NGF (Sigma–Aldrich; St. Louis MO), 5 mg/kg fluoxetine (Eli Lilly and Co.; Indianapolis, IN), or 5 mg/kg amitriptyline (Astra Zeneca Pharmaceuticals LP; Wilmington, DE) for 14 days. The control group received 0.2 ml of the saline alone. Animals were weighed and their health and behavior were recorded daily. At the end of the 14 days of treatment, 24 h after the last application of drugs, rats were euthanized by decapitation, brains were removed and immersed into ice cold saline. Samples of the amygdala/hippocampus were taken from each brain. The samples were immediately placed in individual tubes containing RNAlater (Sigma–Aldrich, St. Louis, MO) and held at 4 °C for four days before transferring to −80 °C freezer. All procedures involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Louisiana Tech University in compliance with the guidelines established by the National Institute of Health.

2.2. RNA extraction and RNA quality control

Brain sections preserved in RNAlater were removed from the original tubes and placed into fresh RNAase-free eppendorff tubes. Tissue samples were mechanically homogenized by using pellet pestles in TRIZOL reagent (1 ml TRIZOL for 200 mg tissue) (Sigma–Alrich, Saint Louis, MO). An additional isolation step was performed according to the supplier's instructions due to relatively high fat, protein and polysaccharide content of the brain samples. Briefly, following homogenization, the homogenate was centrifuged at 12,000*g* for 10 min at 2–8 °C. The supernatant was transferred to a new RNAase-free tube and the pellet was discarded. For the phase separation step, 200 μl of chloroform were added into each tube and vigorously vortexed for 15 s. Samples were incubated at room temperature for three minutes before centrifugation at 12,000*g* for 15 min at 2 to 8 °C. The aqueous phase of each sample was transferred to a fresh tube. RNA was precipitated by adding 0.5 ml isopropyl alcohol per 1 ml TRIZOL. Samples were incubated at 15–30 °C for ten minutes and centrifuged at no more than 12,000*g* for 10 minutes at 2 to 8 °C. RNA pellets were washed twice with 1 ml 75% ethanol by successive mixing and centrifugation at 7500*g* for 5 minutes at 2 to 8 °C. At the end of the procedure, RNA pellets were air-dried for 5–10 minutes and dissolved in RNAase-free water.

Assessment of RNA integrity was done using an RNA LabChips® kit and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). This analysis yielded results that indicated RNA extracted from the brain samples was in good quality and suitable for PCR Array technology.

2.3. RT-PCR and PCR

Prior to the RT PCR step, 1 μg total RNA was treated by gDNA elimination buffer and incubated in a 42 °C water-bath for five minutes in order to digest possible gDNA contamination. Following the elimination step, complementary DNA was synthesized using a RT^2 First Strand Kit (C-03) in accordance with the protocol provided by the manufacturer (SABiosciences, Frederick, MD). Then, cDNA synthesized from 1 μg total RNA was combined with RT^2 Real-Time SYBR Green/ROX PCR master mix (PA-012) provided by the supplier and loaded onto a 96 well RT^2 Profiler PCR standard-Array (PARN-060). PCR reactions were executed by utilizing the ABI 7900 Real-Time machine at the Brown University Center for Genomics and Proteomics Core Facility.

2.4. Analysis

PCR Array data analysis was performed on five rats per treatment group and C_t (Cycle threshold) values greater than 35 were excluded from the data analysis. The PCR array data were analyzed using an online analysis tool provided by the supplier of the PCR arrays (SABiosciences, Frederick, MD) (http://www.sabiosciences.com/pcrarraydataanalysis.php). Briefly, the data were normalized by subtracting the average C_t value of four housekeeping genes from the C_t values of drug treated and saline only samples within the same brain region. Housekeeping genes used for normalization were Actb (Actin, beta), Ldha (Lactate dehydrogenase A), Rplp1 (Ribosomal protein, large, P1) and Hprt (Hypoxanthine–guanine phosphoribosyl transferase). Normalized values were designated as ΔC_t values for each gene and $\Delta\Delta C_t$ values were obtained by subtracting ΔC_t values of saline treated samples from the ΔC_t values of drug treated samples for each gene. In order to calculate the relative fold change in mRNA level of each gene, the formula $2^{-\Delta\Delta Ct}$ was utilized, representing the amount of the expression of each gene in the drug treated sample to the saline control sample. If the values of relative fold change were less than one, $-1/2^{-\Delta\Delta Ct}$ formula was applied to the value for better comparison purposes. Genes that yielded a p-value of <0.05 were considered to display statistical significance for the study.

3. Results

3.1. Changes in gene expression in NGF-treated rats compared with saline controls

Compared to saline-only treated rats, expression of eight genes were significantly changed ($p \leqslant 0.05$) in amygdala/hippocampus following treatment with NGF (Table 1). Interestingly, out of the 84 genes we have screened in the amygdala/hippocampus brain region, NGF uniquely depressed the expression of eight genes, including Drd5, Prokr1, Htr3a, Chrna5, Maoa, Chrnb4, Sstr3, Cckar.

3.2. GABAergic gene expression

Although not meeting a $p \leqslant 0.05$ threshold in each case, there was a tendency for all three study medications, more significantly seen in amitriptyline, to alter GABAergic gene expression relative to levels in saline-treated controls (Table 2). NGF down-regulated the levels of Gabra3 and Glra2 while amitriptyline up-regulated the expression levels of Gabra1, Gabra4, Gabra2. Fluoxetine lowered the expression of Gabrg1 and up-regulated Gabrd expression.

3.3. Similarities between three drugs

There were commonalities in gene expression between NGF and the two antidepressant medications: NGF and amitriptyline both down-regulated the level of Prokr1 in amygdala/hippocampus while Chrna5 was down-regulated by NGF and fluoxetine. The direction of the change in gene expression was the same in each case (Table 3).

Table 1
Changes in gene expression as a result of NGF treatment compared with saline-treated rats, *p*-value <0.1.

Gene symbol	*p*-Value	Fold change	Gene description
Drd5*	0.02	−1.49	Dopamine receptor D5
Prokr1	0.02	−1.44	Prokineticin receptor 1
Htr3a*	0.02	−1.36	5-hydroxytryptamine (serotonin) receptor 3a
Chrna5	0.0009	−1.25	Cholinergic receptor, nicotinic, alpha polypeptide 5
Maoa	0.004	−1.13	Monoamine oxidase A
Chrnb4	0.05	−1.37	Cholinergic receptor, nicotinic, beta polypeptide 4
Sstr3*	0.05	−1.33	Somatostatin receptor 3
Cckar*	0.05	−1.14	Cholecystokinin A receptor
Gabra3*	0.07	−1.23	Gamma-aminobutyric acid (GABA) A receptor, alpha 3
Glra2*	0.10	−1.49	Glycine receptor, alpha 2 unit
Drd3	0.07	2.11	Dopamine receptor D3

* Genes uniquely regulated by NGF but not Fluoxetine or Amitriptyline.

Table 4
Changes in overall neurotransmitter receptors and regulators gene expression in amygdala/hippocampus brain region in rat *p*-value <0.1.

Gene	NGF	*p*-Value	Fluoxetine	*p*-Value	Amitriptyline	*p*-Value
Cckar	−1.14	0.05				
Drd5	−1.49	0.02				
Prokr1	−1.44	0.02			−1.34	0.03
Htr3a	−1.37	0.02				
Maoa	−1.13	0.004			1.08	0.06
Sstr3	−1.33	0.05				
Drd3	2.11	0.07			2.46	0.002
Gabra3	−1.23	0.07				
Glra2	−1.49	0.10				
Chrna5	−1.25	0.0009	−1.19	0.02		
Chrnb4	−1.37	0.05	−1.39	0.07		
Brs3			−1.50	0.02		
Gpr103			−1.39	0.04		
Gabrg1			−1.11	0.07		
Gabrd			1.2	0.08		
Abat					−1.15	0.005
Ache					−1.21	0.02
Chrnb2					−1.15	0.04
Nmur1					−1.67	0.03
Sstr1					−1.26	0.001
Chat					1.73	0.01
Gabra1					1.30	0.02
Gabra4					1.24	0.009
Gabra2					1.22	0.002
Npy5r					1.36	0.003
Gabrb3					1.11	0.05

Table 2
Changes in GABAergic gene expression by medication, *p*-value <0.1.

Treatment	Gene symbol	*p*-Value	Fold change	Gene description
NGF	Glra2	0.10	−1.49	Glycine receptor, alpha 2 subunit
	Gabra3	0.07	−1.23	Gama-aminobutyric acid (GABA-A) receptor, subunit alpha 3
Fluoxetine	Gabrd	0.08	1.23	Gama-aminobutyric acid A receptor, delta
	Gabrg1	0.07	−1.11	Gama-aminobutyric acid A receptor, gamma 1
Amitriptyline	Gabra1	0.02	1.30	Gama-aminobutyric acid A receptor, alpha 1
	Gabra4	0.0094	1.24	Gama-aminobutyric acid (GABA-A) receptor, subunit alpha 4
	Gabra2	0.002	1.22	Similar to Gama-aminobutyric acid receptor alpha-2 subunit precursor (GABA(A))
	Gabrb3	0.05	1.11	Gama-aminobutyric acid (GABA-A) receptor, subunit beta 3

Table 3
Genes regulated by NGF and the two antidepressant medications, *p*-value <0.1.

Genes symbol	NGF	*p*-Value	Fluoxetine	*p*-Value	Amitriptyline	*p*-Value
Prokr1	−1.44	0.02	–	–	−1.34	0.037
Chrna5	−1.25	0.0009	−1.19	0.027	–	–
Maoa	−1.13	0.004	–	–	−1.08	0.06
Chrnb4	−1.37	0.05	−1.39	0.07	–	–
Drd3	2.11	0.07	–	–	2.46	0.002

Prokr1 – Prokineticin receptor 1; Chrna5 – Cholinergic receptor, nicotinic, alpha polypeptide 5; Maoa – Monoamine oxidase A; Chrnb4 – Cholinergic receptor, nicotinic, beta polypeptide 4, Drd3 – Dopamine receptor D3.

3.4. Gene expression changes unique to NGF

Four out of the 84 genes we screened were specifically regulated by NGF but not the two well known antidepressant drugs as compared with saline-treated controls. The four genes with significant ($p \leqslant 0.05$) decreases in gene expression by NGF include: Drd5, Htr3a, Sstr3 and Cckar (see those genes marked with an asterisk in Table 1).

3.5. Changes in gene expression induced by three medications in amygdala/hippocampus

This study screened a total of 84 genes involved in rat neurotransmitter receptors and regulators. Among the three medications, amitriptyline caused the most activity by changing the levels of thirteen genes in the amygdala/hippocampus region of rat brain. With eight genes regulated, NGF followed amitriptyline. Treatment with fluoxetine changed the expression of three genes. A comprehensive list of genes with significantly ($p \leqslant 0.05$) altered expression across the three study medications is listed in Table 4 along with the direction of change in gene expression relative to saline controls.

4. Discussion

Expression of neurotransmitter regulator and receptor genes was assessed in the brain regions most frequently implicated in the neurobiology of depression. Numerous genes were identified as having altered transcription when comparing NGF-treated rat brains to saline-treated rat brains. Given the intent of this study to understand the differences and similarities between NGF and two antidepressant medications, the focus of this discussion will be on the data presented above comparing and contrasting these medications to saline-treated controls. The results of this study suggest that NGF does share some pharmacological properties with fluoxetine and amitriptyline albeit not the targets usually associated with antidepressant efficacy. The congruence of NGF treatment with the two antidepressant medication's effects on GABAergic gene expression is consistent with the evidence that GABA may be a critical substrate for the hippocampal neurogenesis critical to depression treatments (Luscher et al., 2011). Although all three treatments altered the expression of GABAergic receptor subunits, there was no consistent pattern in these changes that might suggest a common pathway. NGF had a similar effect as fluoxetine on reducing the expression of the cholinergic gene CHRNA5. NGF was similar to amitriptyline with regard to the impact on decreasing prokineticin receptor 1 (PROKR1) expression.

By contrast, NGF did have actions unique to the two medications in the amygdala/hippocampus. Significant decreased expression of dopamine D5 receptor (DRD5), serotonin receptor 3A (HTR3A), Somatostatin receptor 3 (SSTR3) and Cholecystokinin A receptor (CCKAR) was found relative to saline-treated controls. The lack of a selective ligand for Dopamine D5 receptors has hampered study of this aspect of the dopaminergic system; however, it is noteworthy that dopamine D5 receptors couple with GABA A receptors (Liu et al., 2000) and the altered gene expression may reflect NGF's impact on the GABAergic system. The 5HT3 receptor is a common target of antiemesis drugs (e.g., ondansetron), although there is also preclinical evidence that manipulating this system may impact anxiety, learning and attention (Bhatnagar et al., 2004; Harrell and Allan, 2003). Possibly consistent with these findings is the report that the 5HT3 antagonist ondansetron has antidepressant effects in the forced swim and tail suspension tasks (Ramamoorthy et al., 2008). Moreover, variation in the 5HT3A gene predicts antidepressant response in depressed Japanese patients (Kato et al., 2006) and different classes of antidepressants act as antagonists at 5-HT3A receptors (Eisensamer et al., 2003) suggesting an alternative mechanism for these medications than the traditional view of serotonin transporter involvement.

SSTR3 is implicated in a number of functions but has been found to inhibit dopamine D1-stimulated cAMP in a manner that suggests that it is coupled with adenylyl cyclase (Yamada et al., 1992). It is possible that alterations in second messenger systems related to dopaminergic tone may impact major depression but there is no evidence of differential expression of D1 receptors in post mortem studies of suicide victims and controls (Dunlop and Nemeroff, 2007). The relationship between this system and depression is not unknown as somatostatin 2 and 3 receptor agonists result in antidepressant-like effects in the forced swim test (Engin and Treit, 2009). CCKAR, on the other hand, has primarily been a target for understanding obesity (Inoue et al., 1997). Despite often suggestive findings, none of the four genes significantly altered by NGF (but not by the two established antidepressants) as compared with the saline controls is presently considered a primary candidate for depression etiology or antidepressant action. Potential interpretations of these results include the possibility that these represent novel pathways for NGF's antidepressant properties. Such findings may also signify true effects of chronic NGF administration that are unrelated to depressive pathology.

It is notable that all but two genes (i.e., PROKR1 and SSTR3) with significantly altered NGF-induced expression in this study are either primary targets of medications for alcohol and other drug dependence (see Kenna et al., 2004a,b for reviews related to 5HT3 and GABA-A receptors) or genes related to addiction etiology (e.g., CHRNA5 and CHRNB4 in Wang et al., 2009; DRD3 and DRD5 in Le Foll et al., 2009; CCKAR in Pommier et al., 2002; MAO-A in Saraceno et al., 2009; and GLRA2 in Yevenes et al., 2010) raising the possibility that NGF might be investigated as a potential treatment for alcohol dependence or other addictions especially in the case of co-morbid addiction and depression. Indeed, reduced levels of serum NGF have been reported in alcohol dependent individuals (Yoon et al., 2006) and those who abuse other drugs of abuse (Angelucci et al., 2007, 2008). The homology between the CHRNA5 and 5HT3 receptors and resultant overlapping pharmacology further support this possibility as nicotine has known antidepressant properties and odansetron (5HT3 antagonist) is under active investigation for the treatment of alcohol dependence. Finally, it remains a possibility that these findings are false positive results due to the large number of tests conducted especially given the relatively small changes in gene expression.

NGF is a large molecule that does not appear to readily cross the blood brain barrier. However, there is experimental evidence for transport of NGF between the peripheral and central compartments (Poduslo & Curran, 1996). The present study was conducted to determine whether chronic subcutaneous NGF administration might result in altered gene expression in brain. We are unable to attribute any such changes in brain gene expression to either transported NGF or as an end result of a peripheral NGF action that has downstream central effects through a yet-to-be-determined mechanism. Interestingly, Lewin et al. (1994) provide evidence of both a peripheral and central hyperalgesic effect after a single systemic NGF injection—thereby providing some support for a cross-compartment effect of peripheral NGF administration as suggested by the data presented here.

Future extensions of the present study might include empirically testing whether NGF shows antidepressant properties in juvenile rats such as those used in this study although other antidepressant medications such as serotonin reuptake inhibitors and tricyclic antidepressants do not have acute effects on the forced swim test (see Pucilowski and Overstreet, 1993). Other directions might include the use of an inert peptide as a control instead of sal-

ine to address the possibility of a non-specific immune reaction, validating the array-identified gene expression changes with follow-up qPCR assays of the individual genes, and the use of microdissection techniques to separate the brain structures in order to provide more specific gene expression changes in the respective regions.

Additionally, the nature of this investigation focused on a mechanism of NGF's antidepressant action as being related to typical neurotransmitters implicated in psychiatric neurobiology. As such, this screen of gene expression products was not an exhaustive effort and may not have identified additional differences in expression of genes unrelated to the neurotransmitter regulator and receptor genes surveyed using this technology. It is notable that the genes identified as having differential expression in this study are not necessarily the same as those in other studies using similar medications, dosing, and brain regions (e.g., Drigues et al. 2003), this may relate to medication-induced gene expression changes that are specific to immature cells and obscured when whole brain regions are examined together (Yamada et al., 2005). Indeed this initial endeavor should be followed up with a more focused investigation of much more narrowly defined sub-regions of the gross anatomy examined here. Also the age of the rats utilized in this study may have resulted in differential findings than if adult rats had been studied. The administration of NGF in the developing brain may have markedly different effects in adult rats and this possibility should be tested empirically. Moreover, consistent with all gene expression research, the changes in gene products reported here may or may not be reflected in changes in protein levels. Nevertheless, the findings reported here represent an important and critical first step in identifying a potential mechanism for the observed antidepressant effects of NGF.

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