OPEN

Citation: Transl Psychiatry (2015) 5, e511; doi:10.1038/tp.2015.11

www.nature.com/tp

ORIGINAL ARTICLE

MicroRNA regulation of central glial cell line-derived neurotrophic factor (GDNF) signalling in depression

M Maheu[1,2], JP Lopez[1,3], L Crapper[1,2], MA Davoli[1], G Turecki[1,2,3,4] and N Mechawar[1,2,4]

Although multiple studies have reported that peripheral glial cell line-derived neurotrophic factor (GDNF) is reduced in depression, cerebral GDNF signalling has yet to be examined in this condition. Here, we report an isoform-specific decrease in GDNF family receptor alpha 1 (GFRA1) mRNA expression, resulting in lowered GFRα1a protein levels in basolateral amygdala (BLA) samples from depressed subjects. Downregulation of GFRα1a was associated with increased expression of microRNAs, including miR-511, predicted to bind to long 3′ untranslated region (3′-UTR)-containing transcripts (GFRA1-L) coding for GFRα1a. Transfection of human neural progenitor cells (NPCs) with a miR-511 mimic was sufficient to repress GFRA1-L/GFRα1a without altering GFRα1b, and resulted in pathway-specific changes in immediate early gene activity. Unexpectedly, GFRα1a knockdown did not reduce NPC responses to GDNF. Rather, it greatly enhanced mitogen-activated protein kinase signalling. This effect appeared to be mediated by GDNF/soluble GFRα1/neural cell adhesion molecule binding, and substituting the soluble GFRα1a/GFRα1b content of miR-511-transfected NPCs with that of controls rescued signalling. In light of previous reports suggesting that GFRα1b can inhibit GFRα1a-induced neuroplasticity, we also assessed the association between GFRα1 and doublecortin (DCX; a hyperplastic marker) in human BLA. Although controls displayed coordinated expression of GFRα1a and b isoforms and these correlated positively with DCX, the only significant association observed among depressed subjects was a strongly negative correlation between GFRα1b and DCX. Taken together, these results suggest that microRNA-mediated reductions of GFRα1a in depression change the quality, rather than the quantity, of GDNF signalling. They also suggest that central GDNF signalling may represent a novel target for antidepressant treatment.

Translational Psychiatry (2015) **5,** e511; doi:10.1038/tp.2015.11; published online 17 February 2015

INTRODUCTION

Despite a growing body of literature implicating impaired cellular plasticity within limbic brain regions in depression, little is known about the exact mechanisms that underlie these dysfunctions. Within the basolateral amygdala (BLA), stress results in considerable neuroplastic adaptation; animal studies have consistently reported that dendritic arborization and spine density are increased in principal neurons following chronic stressors,[1–3] whereas acute stress results in delayed increases in spine density in the absence of dendritic remodelling, or in combination with dendritic retraction.[4–6] These morphological changes, in turn, underlie stress-induced behavioural adaptations including increased anxiety, which can be blocked by experimental manipulations that inhibit structural plasticity in the BLA.[7] Thus, in general, stress is marked by adaptive neuronal remodelling in the BLA and anxiety-like behaviour. However, under certain pathological conditions, the positive association between the amount of structural plasticity that occurs in the BLA in response to stress, and the extent to which the organism's behaviour is affected by that stress, is not maintained. Instead, negative outcomes are associated with an absence of structural remodelling.[5,8]

Neurotrophins have long been recognized as key mediators of structural plasticity, and a large number of studies support their role in depression and as candidate targets for effective pharmacological intervention.[9] Yet, with the exception of brain-derived neurotrophic factor, few investigations into how these molecules are regulated in the depressed human brain have been published. Among these, glial cell line-derived neurotrophic factor (GDNF) signalling has, until recently, been explored principally in the context of Parkinson's Disease. However, a growing body of work suggests that GDNF may also have a crucial role in affective regulation. Depression has been associated with reduced blood and plasma expression of GDNF in adolescents,[10] adults[11–13] and the elderly,[13,14] whereas treatment with pharmacological antidepressants[15] or electroconvulsive therapy[16] increase its expression. To the best of our knowledge, no study of the effects of antidepressants on GDNF levels in the human brain has been completed but, in rodents, interventions that reduce depressive symptoms also enhance central GDNF expression.[17,18] Genetic and epigenetic contributions to GDNF functioning in depression, and following stress or antidepressant treatment, likewise support the idea that central GDNF signalling regulates affect in both rodents[19] and humans.[20,21] Taken together, these data suggest that, as in the periphery, the regulation of central GDNF signalling is likely impaired in depression. What these deficits may mean for neuronal function, however, remains unclear.

The downstream effects of GDNF signalling are regulated through the expression of the ligand itself, as well as by GDNF's interactions with its targets.[22,23] GDNF's principal receptor, the

[1]McGill Group for Suicide Studies, Douglas Mental Health University Institute, McGill University, Montreal, QC, Canada; [2]Integrated Program in Neuroscience, McGill University, Montreal, QC, Canada; [3]Department of Human Genetics, McGill University, Montreal, QC, Canada and [4]Department of Psychiatry, McGill University, Montreal, QC, Canada. Correspondence: Dr N Mechawar, Department of Psychiatry, Douglas Mental Health University Institute, McGill University, 6875 LaSalle Boulevard, FBC Pavilion, Verdun, QC, Canada H4H1R3.
E-mail: naguib.mechawar@mcgill.ca
Received 12 December 2014; accepted 19 December 2014

GDNF family receptor alpha 1 (GFRα1), exists in both a free-floating, soluble form as well as in an insoluble form that is anchored to the lipid membrane of cells by a glycosylphosphatidylinositol (GPI) link. As a result, activation of GPI-anchored GFRα1 induces signalling within the cell to which it is bound (*cis* signalling), whereas soluble GFRα1 released into the extracellular space may act upon neighbouring cells (*trans* signalling). As GFRα1 possesses no transmembrane domain, intracellular signalling by the GDNF/GFRα1 complex is induced via recruitment of one of two co-receptors: REarranged during Transfection (RET) or intracellular domain-containing isoforms of neural cell adhesion molecule (NCAM). Although there is some overlap in the effectors used by RET and NCAM, and both have been shown to induce mitogen-activated protein kinase (MAPK) and Akt activity, these receptors mediate different cellular processes and have distinct effects on structural plasticity in different cell populations.[23–26]

The goal of the present study was to explore whether expression of GDNF signalling molecules is altered in the BLA of individuals suffering from depression, and if so, identify both the upstream regulators and downstream effects of these changes.

MATERIALS AND METHODS

Human brain samples

Informed written consent was acquired from next of kin for all subjects included in this study, and all work was conducted with the approval of the Douglas Hospital Research Ethics Board. Frozen postmortem BLA samples from individuals having died by suicide during a major depressive episode (depressed subjects; $n = 16$) and age- and gender-matched, psychiatrically healthy, sudden-death controls (CTRL; $n = 21$) were provided by the Suicide section of the Douglas-Bell Canada Brain Bank (www.douglasbrainbank.ca; Table 1). Whenever possible, microRNA, messenger RNA (mRNA) and protein were collected from each subject (Supplementary Table 1). For additional information on human brain samples, see Supplementary Materials.

Quantitative real-time PCR

For details regarding total RNA isolation, see Supplementary Materials. For mRNA, quantitative real-time PCR (qRT-PCR) samples were run in quadruplicate or quintuplicate using either TaqMan or SYBR/IDT probes (Integrated DNA Technologies, Coralville, IA, USA). MicroRNA expression was assessed using TaqMan probes, with samples run in quadruplicate (see Supplementary Table 2 for a complete list of primers used). PCRs were run using the ABI 7900HT Fast Real-Time PCR System, and data analysed with the Sequence Detection System software (Applied Biosystems, Burlington, ON, Canada). RNA expression levels were determined using the Absolute Quantitation standard curve method, with GAPDH and RNU6B as previously validated endogenous controls.[27]

Immunoblotting

For details regarding total protein isolation and immunoblotting, see Supplementary Materials. Protein samples were resolved through SDS-polyacrylamide gel electrophoresis and membranes probed for proteins of interest (see Supplementary Table 3 for a complete list of antibodies used). Signals were revealed with ECL against film and protein quantity expressed as the optical density of target bands/β-actin.

Immunohistochemistry

Three uncharacterized fresh-fixed BLA samples were provided by the Suicide section of the Douglas-Bell Canada Brain Bank for qualitative immunohistochemistry. For details, see Supplementary Materials.

MicroRNA target prediction analysis and validation

Candidate microRNAs capable of binding GFRA1 mRNA were identified using five microRNA target prediction databases: miRWalk, microRNA.org, RNA22, RNA Hybrid and TargetScan.[28–32] Only microRNAs predicted to bind GFRA1 by at least three of five databases and confirmed to be expressed in human brain as previously described[27,33] were selected for validation using qRT-PCR.

MicroRNA transfections

For details regarding human neural progenitor cell (NPC) growth, maintenance and transfection, see Supplementary Materials. NPCs were transfected with a miR-511 mimic using HiPerFect transfection reagent and collected either 48 h, 72 h or 1 week later.

Table 1. Brain sample information and clinical characteristics of psychiatrically healthy controls (CTRL) and depressed subjects (DS)

	CTRL (N = 21)	*DS (N = 16)*	P-*value*
Sex	17 M; 4F	10 M; 6F	0.274
Age (±s.d.)	43.3 (18.83)	41.75 (16.25)	0.790
PMI (±s.d.)	25.05 (15.12)	27.78 (24.92)	0.682
Brain pH (±s.d.)	6.49 (0.30)	6.58 (0.27)	0.348
Axis 1 disorder (*n*)	Nil (21)	MDD (16)	NA
Alcohol/drug dependence (*n*)	Alcohol dependence (2)	Alcohol dependence (1) Drug dependence (1) Alcohol and drug dependence (2)	NA
Toxicology results at time of death (*n*)	Alcohol (1)	Alcohol (2)	
	Diazepam (benzodiazepine) (1)	Temazepam (benzodiazepine)+codeine (opiate) +acetaminophen (1)	NA
	Oxazepam (benzodiazepine)+sodium thiopenthal (barbiturate) (1) Dextromethorphan (1)	Venlafaxine (SNRI)+lorazepam (benzodiazepine) +acetaminophen (1)	
Cause of death (*n*)	Cardiovascular failure (7) Car accident (6) Cancer (3) Stroke (1) Accidental hanging (1) Unknown natural causes (2) Surgical/postoperative complications (1)	Hanging (9) CO intoxication (2) CO_2 intoxication (2) Intoxication (1) Jump from height (1) Gunshot wound (1)	NA

Abbreviations: CO, carbon monoxide; CO_2, carbon dioxide; MDD, major depressive disorder; NA, not applicable. Groups were matched for sex, age, postmortem interval (PMI) and brain pH, and did not significantly differ in any of these measures. Two-tailed Student's *t*-tests (age, PMI and brain pH) and Fisher's tests (sex) were used for group comparisons.

GDNF administration

Seventy-two hours following transfection, human NPCs were administered either 1 ng ml^{-1} or 50 nm ml^{-1} of GDNF (PeproTech, Dollard des Ormeaux, QC, Canada). GDNF was added either directly to existing media (for studies of GDNF signalling in *cis*+*trans*), or diluted in fresh media (for studies of GDNF signalling in *cis*). In the latter case, the old media was removed from wells immediately beforehand, and cells briefly rinsed with phosphate-buffered saline warmed to 37 °C before the addition of the new, GDNF-containing media. After either 5 or 60 min, media were removed from wells and cells rinsed with phosphate-buffered saline warmed to 37°C before collection.

Statistical analyses

Statistical analyses were performed using PASW Statistics 18 (Statistical Product and Service Solutions, Chicago, IL, USA) and GraphPad Prism v. 6.0 (GraphPad Software, La Jolla, CA, USA). Graphs were produced using GraphPad Prism v. 6.0 and all measures are expressed as mean ± s.e.m. Shapiro Wilk and Levene's tests were employed to assess data normality and equality of variance, and outlier detection was completed using the 1.5 interquartile range method. Student's *t*-tests were used for two-group comparisons unless data were found to be nonparametric, in which case Mann–Whitney *U*-tests were used. For comparisons between three or more groups, data were assessed using one- and two-way analyses of variance followed by Bonferroni's *post hoc* tests, and *P*-values on graphs reflect significance of *post hoc* tests. All correlations are pairwise.

RESULTS

Protein and gene expression of GDNF signalling molecules in the BLA of depressed subjects

Our initial examination of the protein expression of GDNF, GFRα1, NCAM and RET revealed decreased expression of GFRα1 in the BLA of depressed subjects (Figures 1a–d). However, when various isoforms of the GFRA1 gene were assessed, no difference in the mRNA expression of total GFRA1, truncated GFRA1 or combined full-length GFRA1 receptors was observed (Figures 1e–g), nor did GFRA1 mRNA expression correlate with protein levels (Figure 1h).

The human GFRA1 gene encodes two full-length isoforms: GFRα1a, which possesses an additional 15 bp exon (exon 5), and GFRα1b. GFRα1a and b were expressed at a ratio of ~1 to 2 in the human BLA (Supplementary Figure 2). Aside from coding regions, these splice variants are believed to display considerable heterogeneity in 3′-UTR length; Ensembl (www.ensembl.org) estimates range from 634 to 7465 bp (Figure 1i). qRT-PCR using primers designed to target the 3′ end of transcripts containing the long, 7465 bp 3′-UTR (hereafter called GFRA1-L) revealed decreased expression of this transcript in the BLA of depressed subjects (Figure 1j), and mRNA levels of GFRA1-L correlated positively with GFRα1 protein (Figure 1k). Immunohistochemical localization of GFRα1 revealed that the receptor was expressed solely by neurons. GFRα1 receptors appeared to be localized primarily on the soma and proximal dendrites on cells displaying both pyramidal and aspinious/stellate morphology (Figure 1l).

MicroRNA regulation of GFRA1-L in human BLA

Given that expression of mRNAs possessing shorter 3′-UTRs was spared in depressed subjects, we hypothesized that GFRA1-L's much longer 3′-UTR might provide additional opportunity for microRNAs to bind to this transcript and downregulate its expression. Using open source target prediction applications[28–32] to identify candidate microRNAs that might bind to GFRA1-L, and following an initial screening to confirm their expression in human brain tissue,[27,33] expression of miR-875-5p, miR-511 and miR-340 was assessed in the BLA using qRT-PCR. Although miR-875-5p was undetectable in the human BLA, depressed subjects displayed significantly increased miR-511 levels and a strong tendency toward increased miR-340 expression as compared with controls (Figures 2a and b). Furthermore, miR-340 and -511 correlated positively and with one another, and negatively with GFRα1 protein levels (Figures 2c–e).

miR-511 regulation of GFRα1a/GFRA1-L in human NPCs

In silico predictions of miR-511 binding to GFRA1-L's 3′-UTR include up to 10 potential binding regions (BR).[28–32] To test whether miR-511 was sufficient to specifically reduce GFRA1-L expression, human NPCs were transfected with a miR-511 mimic. qRT-PCR using three primer sets (called BR1, BR8/9 and BR10) that overlap selected miR-511 BRs were then used to quantify gene expression. BR1 primers were designed to amplify a low-affinity, 6mer site with folding energy of < -20 kcal mol^{-1} that is present on GFRA1-L as well as other GFRA1 transcripts. BR8/9 (comprising two predicted BRs including one < -20 kcal mol^{-1}, 7mer-m8 site) and BR10 (< -30 kcal mol^{-1}, 8mer site) are high-affinity BRs unique to GFRA1-L (Figure 2f). MiR-511 transfection reduced expression of BR8/9 and BR10-containing mRNAs, whereas BR1 expression was unchanged (Figures 2h–j), suggesting that miR-511 specifically repressed expression of GFRA1-L, while leaving expression of GFRα1b-producing mRNAs intact. To confirm that reduced expression of GFRA1-L did indeed reflect down-regulation of GFRα1a transcription, we next assessed expression of the GFRα1a-specific exon 5. GFRα1a exon 5 mRNA was significantly reduced in miR-511 transfected cells (Figure 2g), and correlated positively with BR8/9 and BR10 expression levels (Supplementary Figure 3). These data support the hypothesis that increased expression of miR-511 results in a selective repression of GFRA1-L mRNA and GFRα1a protein.

Association between GFRα1a expression and doublecortin expression in the human BLA

Few studies have examined the role of isoform-specific GFRα1 signalling, but evidence suggests that GFRα1a and GFRα1b elicit different downstream effects and have opposing roles in certain aspects of structural plasticity.[34,35] Although GFRα1a has been shown to enhance neurite outgrowth in stably transfected neuro2D cells exposed to GDNF, co-transfection with GFRα1b inhibits this process.[35] These findings suggest that reduced expression of GFRA1-L/GFRα1a in the human BLA may be associated with, or even contribute to, reductions in neuroplasticity.

Doublecortin (DCX) is a microtubule-associated neuroplastic protein expressed both by neuroblasts and by neurons born during early development that retain an immature/hyperplastic phenotype and which appear to function similarly to new, adult-born neurons in brain regions that do not retain neurogenic capacity throughout life.[36–38] Previous data from our lab suggest that BLA expression of DCX is altered in depression, and that reduced expression of this protein may be associated with worse outcomes among depressed individuals.[8] When DCX levels were re-assessed in tandem with GFRα1 protein expression in the human BLA, we found that these two molecules correlated significantly and positively with one another ($R^2 = 0.407$, $P = 0.001$; Figure 3a). This correlation was maintained when control subjects were examined in isolation ($R^2 = 0.577$, $P = 0.004$), but was absent in depressed individuals ($R^2 = 0.042$, $P = 0.569$; Figures 3b and c). Similarly, higher DCX expression was correlated with elevated mRNA expression of the GFRA1-L transcript overall ($R^2 = 0.273$, $P = 0.038$) as well as in the control group ($R^2 = 0.479$, $P = 0.057$), but not among depressed subjects (Figures 3d and e). The same pattern of positive reciprocal associations were found among controls in all pairwise comparisons of the following: DCX protein, GFRα1 protein, GFRA1-L mRNA and even BR1-containing GFRA1 transcripts (Figure 3k). They disappeared altogether among depressed subjects, however. The only significant association detected among depressed subjects was a strongly negative correlation between DCX and non-GFRA1-L BR1-containing

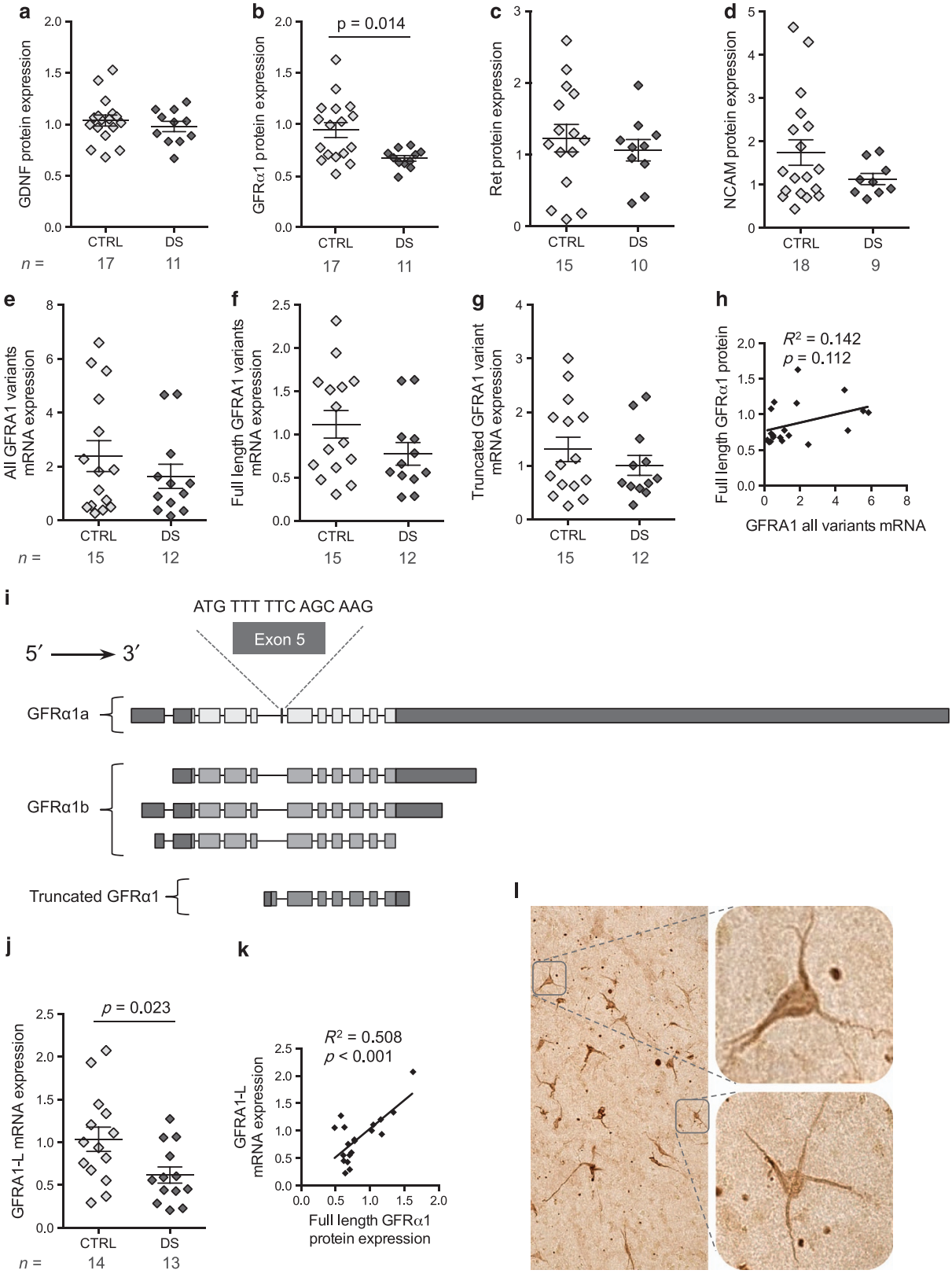
a
GDNF protein expression
CTRL
DS
n = 17 11
b
GFRα1 protein expression
p = 0.014
CTRL
DS
17 11
c
Ret protein expression
CTRL
DS
15 10
d
NCAM protein expression
CTRL
DS
18 9
e
All GFRA1 variants mRNA expression
CTRL
DS
n = 15 12
f
Full length GFRA1 variants mRNA expression
CTRL
DS
15 12
g
Truncated GFRA1 variant mRNA expression
CTRL
DS
15 12
h
Full length GFRα1 protein
R² = 0.142
p = 0.112
GFRA1 all variants mRNA
i
ATG TTT TTC AGC AAG
Exon 5
5′ → 3′
GFRα1a
GFRα1b
Truncated GFRα1
j
GFRA1-L mRNA expression
p = 0.023
CTRL
DS
n = 14 13
k
GFRA1-L mRNA expression
R² = 0.508
p < 0.001
Full length GFRα1 protein expression
l

Figure 1. Expression of GDNF signalling molecules in the basolateral amygdala (BLA). Protein expression of (**a**) GDNF, (**b**) GFRα1, (**c**) RET and (**d**) NCAM in the BLA of depressed subjects (DS) and age- and gender-matched psychiatrically healthy controls (CTRL). Quantitative real-time PCR expression of (**e**) pan-GFRA1 mRNA transcripts, (**f**) full-length GFRA1 mRNA and (**g**) truncated GFRA1 mRNA in the BLA. (**h**) Correlation between pan-GFRA1 mRNA expression and BLA protein levels of GFRα1. (**i**) Proportional schematic representation of GFRA1 transcript variants and subclasses. Lighter boxes represent coding exons; dark boxes represent 5′ and 3′ untranslated regions (UTRs). All exons, including UTRs, are sized proportionally. (**j**) Quantitative real-time PCR expression of GFRA1-L/GFRα1a and (**k**) correlation with BLA protein expression of GFRα1 protein. (**l**) Immunohistochemical localization of GFRα1 in the human BLA. Equality of variance was assessed using Levene's tests, and group comparisons assessed using two-tailed Student's *t*-tests and Mann–Whitney *U*-tests (**a**–**g** and **j**), and Pearson's correlations coefficients (**h** and **k**). All biological replicates (*n*) are displayed on graphs along with the group mean ± s.e.m. GDNF, glial cell line-derived neurotrophic factor; GFRA1, GDNF family receptor alpha 1 gene; GFRα1, GDNF family receptor alpha 1 protein; mRNA, messenger RNA; NCAM, neural cell adhesion molecule.

transcripts ($R^2 = 0.808$, $P = 0.015$; Figures 3i–l). Importantly, these associations did not appear to be mediated by a direct interaction between DCX and miR-511, as miR-511 levels did not correlate with DCX among depressed subjects, nor was 1 week of miR-511 transfection sufficient to decrease DCX expression *in vitro* (Supplementary Figure 4).

Effect of miR-511-mediated GFRα1a knockdown on GDNF signalling: gene transcription

To assess how miR-511-mediated GFRA1-L/GFRα1a reduction affected gene transcription *in vitro*, NPCs were administered GDNF 72 h after miR-511 transfection and the expression of immediate early genes responsive to GDNF[15] measured using qRT-PCR. In control cells, administration of 1 ng ml^{-1} and 50 ng ml^{-1} of GDNF resulted in increasing Fos expression (Figure 4a). Early growth response 1 and 2 (Egr1/2) expression was likewise elevated following 1 ng ml^{-1} GDNF, but did not increase further at higher ligand dosages (Figures 4b and c). MiR-511-transfected NPCs, on the other hand, displayed roughly twofold greater induction of Fos following 1 ng ml^{-1} GDNF administration than did control cells (Figure 4a), and 1.5-fold greater induction following 50 ng ml^{-1}. Fos expression correlated negatively with the ratio of GFRα1a to GFRα1b expressed by NPCs (Supplementary Figure 5a). Conversely, Egr1 and Egr2 did not correlate with GFRα1 expression and remained comparable in both groups, although miR-511-transfected NPCs displayed higher baseline Egr2 activity (Figures 4b and c; Supplementary Figure 5b and c). These data reveal that the capacity of GDNF to induce activation of immediate early genes is not globally suppressed by knockdown of GFRA1-L/GFRα1a, as would be expected by a simple reduction in the number of receptors available for ligand binding. Instead, microRNA-mediated reductions of GFRα1a appear to enhance signalling in a subset of downstream pathways, suggesting that GDNF signalling is qualitatively, rather than quantitatively, altered by miR-511 overexpression.

Effect of miR-511-mediated GFRα1a knockdown on GDNF signalling: Akt and MAPK activity

Although we cannot clearly identify the extent and distribution of soluble GFRα1 in the human BLA, immunohistochemical localization of membrane-bound GFRα1 revealed that its expression was neuron-specific (Figure 1l). RET was expressed only by astrocytes, however (data not shown). Neuronal GDNF signalling in the human BLA is therefore likely mediated by the ubiquitously expressed NCAM molecule, which functions as a co-receptor in both polysialylated and unsialylated forms[39,40] (Supplementary Figure 5a). Screening of human NPCs revealed that, as with human BLA neurons, these cells expressed NCAM, but not RET (Supplementary Figure 5b).

To investigate which signalling pathways are affected by microRNA-mediated decreases in GFRA1-L/GFRα1a expression, NCAM+/RET− NPCs were transfected with a miR-511 mimic and exposed to 1 ng ml^{-1} GDNF. MAPK and Akt activity was then assessed either 5 or 60 min later. Because little is known regarding how GPI-anchored GFRα1/NCAM and soluble GFRα1/NCAM signalling differ, we additionally explored how GDNF signalling in the presence (in *cis* and *trans*) and absence (in *cis*) of soluble GFRα1 might be altered by miR-511 transfection. Thus, GDNF was either added directly into the existing media, or added to fresh media, which was then added to the NPC wells after the old media were removed. (Figure 4d).

In the absence of soluble GFRα1, GDNF administration doubled phospho-Akt expression by 60 min post-GDNF in both groups (Figure 4e), but had no effect on MAPK activation. Conversely, in the presence of soluble GFRα1, GDNF had no impact on Akt activity. Instead, MAPK phosphorylation transiently increased 11-fold in control cells, and over 17-fold in miR-511-transfected cells (Figure 4f). Substituting the cell culture media of untransfected cells with media removed from miR-511-transfected NPCs immediately before GDNF administration—in essence, replacing the control cells's normal complement of soluble GFRα1 receptors with those produced by miR-511-expressing cells—resulted in a slight but nonsignificant increase in MAPK activity in control cells (Figure 4g). However, rescue of soluble GFRα1a levels by substituting the media of miR-511-transfected NPCs with control media before GDNF administration abolished GDNF-induced MAPK overactivity (Figure 4g). These data indicate that miR-511-mediated knockdown of GFRA1-L/GFRα1a greatly enhances MAPK signalling in response to GDNF while exerting minimal effects on Akt activity. Moreover, changes in MAPK signalling appear to be mediated principally by soluble GDNF/GFRα1a/NCAM interactions, whereas maintenance of membrane-bound GFRα1a exerts a protective effect.

DISCUSSION

To the best of our knowledge this is the first assessment of GDNF signalling and regulation in the depressed human brain. Unlike in the periphery, where depression has been associated with altered expression of GDNF,[10–14] we found no change in BLA GDNF levels. Rather, our study revealed that a specific isoform of the GFRA1 gene encoding the GFRα1a receptor is downregulated in depression. Our data further suggest that this repression is mediated by microRNAs, including miR-511, which are capable of specifically targeting GFRA1-L/GFRα1a transcripts while leaving expression of GFRα1b intact. By reducing the ratio of GFRα1a to GFRα1b MAPK, but not Akt, activity was greatly enhanced in NPCs via GDNF/soluble GFRα1/NCAM binding.

Although the means by which reducing the GFRα1a to b ratio facilitates rapid MAPK signalling in NPCs remain unclear, GDNF's differential affinity for the two GFRα1 isoforms represents a strong candidate mechanism. GDNF has been shown to display greater affinity for GFRα1b than it does for GFRα1a and, as a result, GDNF/GFRα1 complexes are more likely to lead to RET phosphorylation at lower ligand concentrations.[34] Even modest reductions in GFRα1a expression may therefore considerably enhance GDNF/GFRα1b binding when GDNF is scarce enough to ensure receptor competition. Importantly, we only observed changes in GDNF-induced MAPK activity when signalling occurred in both *cis* and

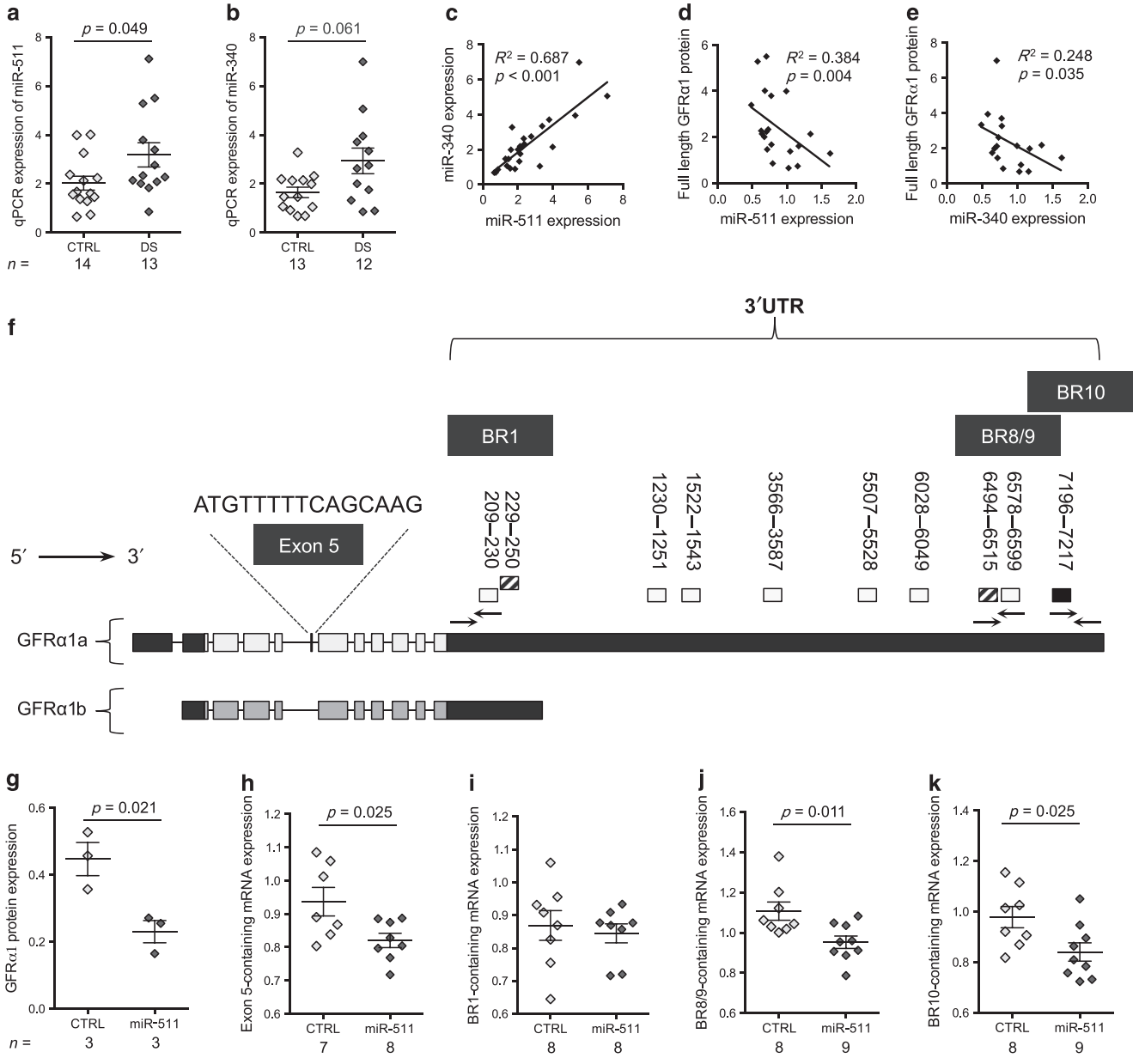

Figure 2. MicroRNA regulation of specific GFRA1 mRNA variants. Expression of (**a**) miR-511 and (**b**) miR-340 in the basolateral amygdala (BLA) of depressed subjects (DS; $n = 12$–13) and matched psychiatrically healthy controls (CTRL; $n = 13$–14). (**c**) Correlation between BLA levels of miR-511 and miR-340. (**d**) Correlation between miR-511 and GFRα1 protein expression in the BLA. (**e**) Correlation between miR-340 and GFRα1 protein expression in the BLA. (**f**) Schematic depiction of predicted miR-511 binding regions (BRs) on GFRα1a- and GFRα1b-coding transcripts. BRs with microRNA seed-region complementarity of at least 7mer-m8 (denoting perfect complementarity of bases 2–8) and a predicted folding energy of < -20 kcal mol^{-1} are shown as striped boxes. BRs with better than 7mer-m8 complementarity and predicted folding energy of < -30 kcal mol^{-1} are depicted as black boxes. Low-affinity BRs, defined as those possessing less than perfect seed-region complementarity, are depicted as grey boxes. Binding sites for primers designed to target BR1, BR8/9 and BR10 are shown as arrows. (**g**) Protein expression of GFRα1 72 h after transfection ($n = 3$). Quantitative real-time PCR expression of (**h**) GFRα1a-specific exon 5, (**i**) BR1, (**j**) GFRα1a-specific BR8/9 and (**k**) GFRα1a-specific BR10 in human neural progenitor cells after 48 h of miR-511-transfection ($n = 7$–9). Equality of variance was assessed using Levene's tests, and group comparisons assessed using two-tailed Student's *t*-tests and Mann–Whitney *U*-tests (**a**, **b** and **g**–**j**) and Pearson's correlations coefficients (**c**–**e**). All biological replicates (*n*) are displayed on graphs along with the group mean ± s.e.m. GFRA1, glial cell line-derived neurotrophic factor (GDNF) family receptor alpha 1 gene; GFRα1, GDNF family receptor alpha 1 protein.

trans; *cis*-only GDNF/GFRα1 signalling elicited Akt phosphorylation, but no MAPK induction in our human NPC model. Although we cannot exclude the possibility that this effect is mediated by GFRα1's interaction with NCAM molecules located within different membrane subdomains (for example, within versus outside of lipid rafts[41,42]), an alternate explanation is that a certain minimum threshold of activation is required to induce MAPK signalling, and that this was achieved when soluble GFRα1 receptors were retained. Soluble GFRα1 titration experiments would clarify whether it is receptor number, or location, that determines the extent to which soluble GFRα1/NCAM induces MAPK activity.

Increasing GFRα1b signalling by decreasing GFRα1a expression likely does more than modulate the gain of GDNF. It probably also changes the message. In addition to differences in their capacity to bind GDNF, the GFRα1 isoforms are functionally distinct. They induce divergent patterns of gene expression when stimulated

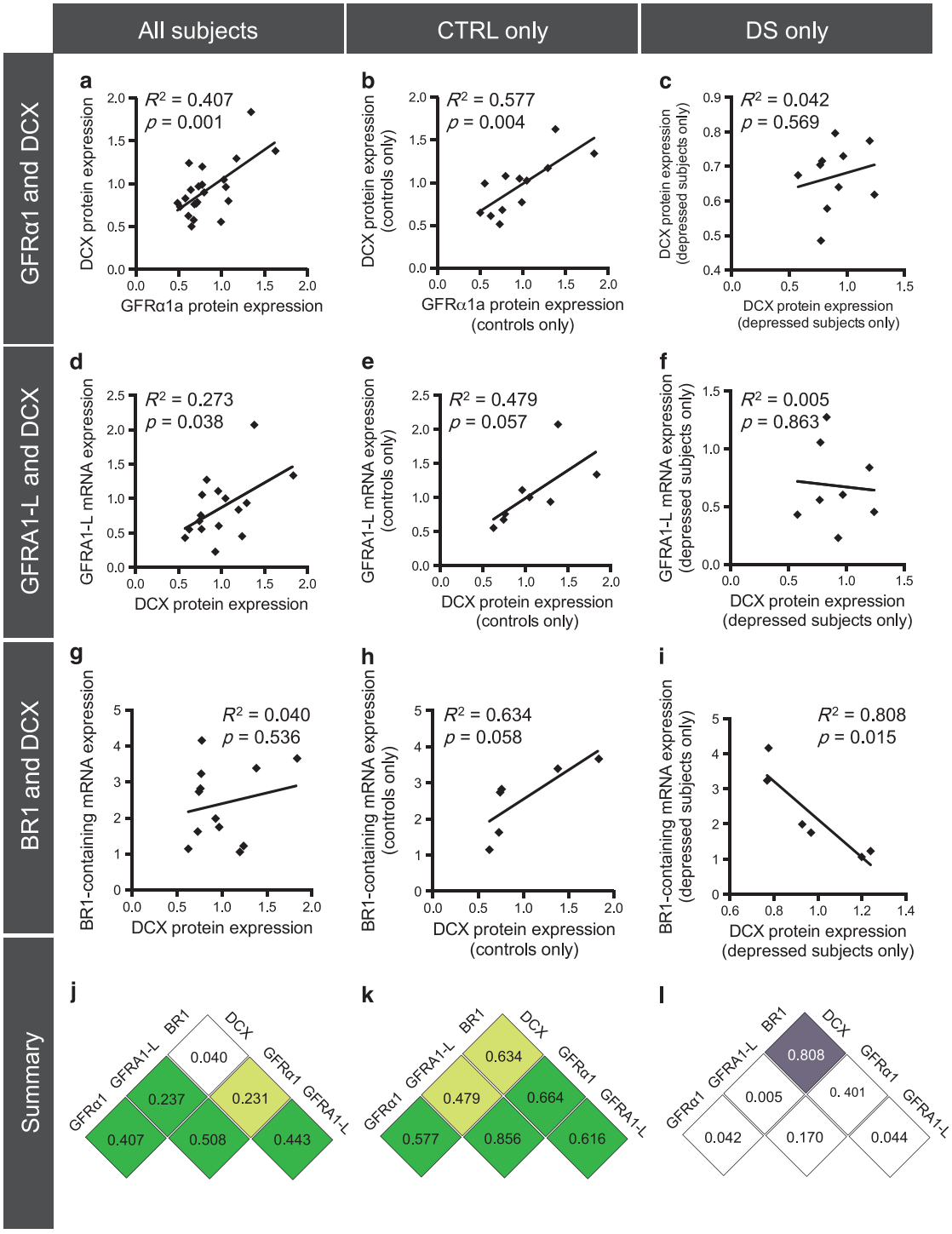

Figure 3. Association between DCX levels and GDNF receptor expression in the human BLA. Correlations between DCX and GFRα1 protein levels in the BLA of (**a**) all subjects, (**b**) controls (CTRL) only and (**c**) depressed subjects (DS) only. Correlations between DCX protein and GFRA1-L mRNA expression in the BLA of (**d**) all subjects, (**e**) in CTRLs and (**f**) in the DS group. Correlations between DCX protein and BR1-containing mRNA expression in the BLA of (**g**) all subjects, (**h**) in CTRLs and (**i**) in the DS group. All pairwise correlations between DCX, GFRα1 protein, GFRA1-L mRNA and BR1-containing mRNA in (**j**) all subjects, (**k**) controls and (**j**) DS subjects. The R^2 value is shown for each comparison, and the statistical significance depicted by color: significant positive correlations ($P < 0.05$) are depicted as dark green boxes, positive statistical trends ($P < 0.1$) are depicted as light green boxes and significant negative correlations are depicted as purple boxes. White boxes denote no significant association between variables. Pearson's correlations coefficients were used for all tests. BR, binding region; DCX, doublecortin; GDNF, glial cell line-derived neurotrophic factor; GFRA1, GDNF family receptor alpha 1 gene; GFRα1, GDNF family receptor alpha 1 protein; mRNA, messenger RNA.

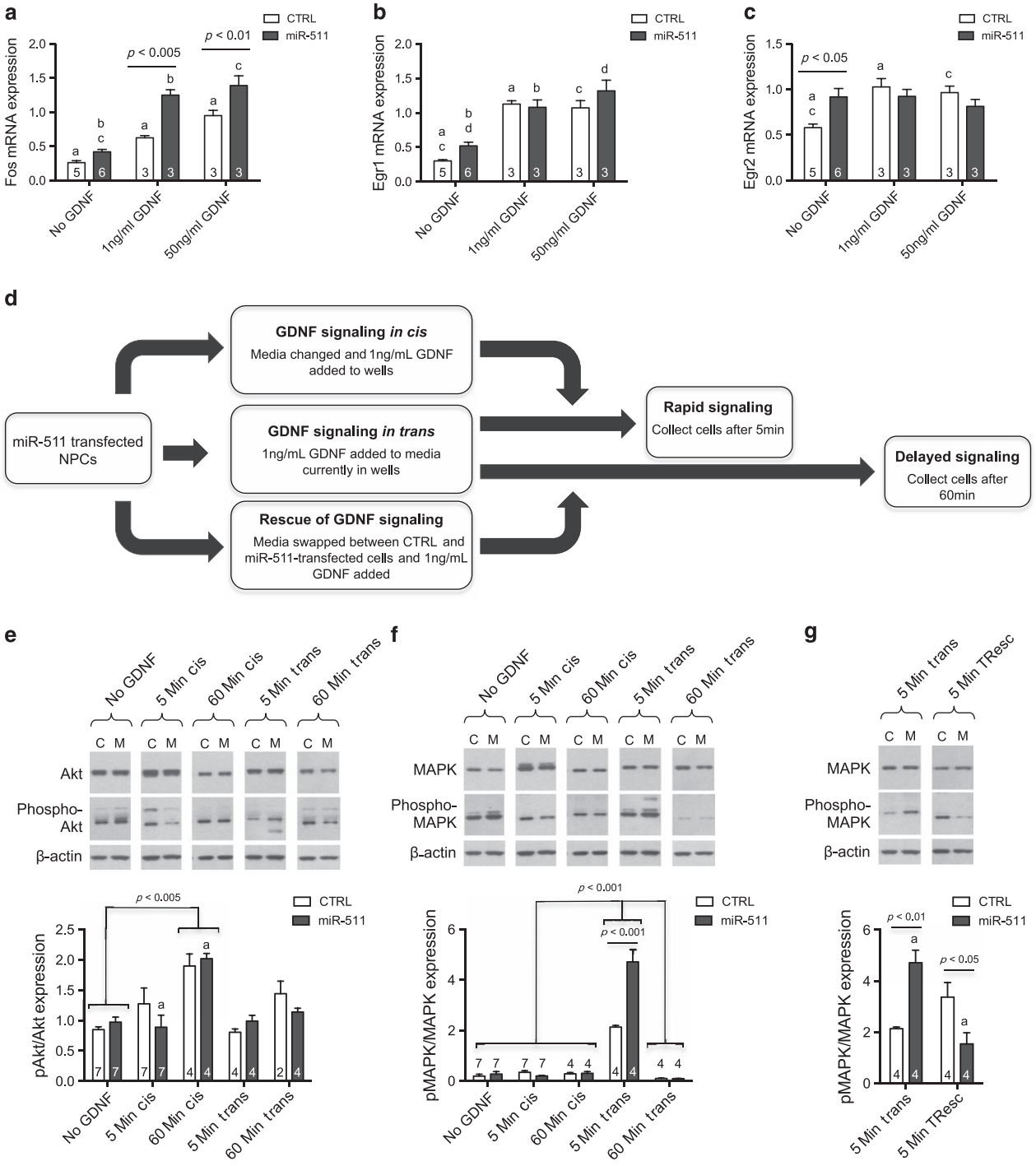

Figure 4. Effects of miR-511 overexpression on GDNF-induced signalling. Expression of immediate early genes (**a**) Fos ($n = 3–6$), (**b**) Egr1 ($n = 3–6$) and (**c**) Egr2 ($n = 3–6$) in miR-511-transfected human neural progenitor cells (NPCs) after treatment with 1 and 50 ng ml^{-1} GDNF. (**d**) Schematic of methodology used to assess GDNF-induced MAPK and Akt signalling. (**e**) Akt activity following 1 ng ml^{-1} GDNF administration in miR-511-transfected human NPCs. ($n = 4–7$) (**f**) MAPK activity following 1 ng ml^{-1} GDNF administration in miR-511-transfected human NPCs ($n = 4–7$). (**g**) Rescue of GDNF signalling in miR-511-transfected NPCs. All numerical data are depicted as mean ± s.e.m. Group differences were assessed using two-way analysis of variance (ANOVA; **b**–**d**) followed by Tukey's and Sidak's *post hoc* tests (**b**–**d**). *n* represents biological replicates. GDNF, glial cell line-derived neurotrophic factor; MAPK, mitogen-activated protein kinase.

and, at least in stably transfected Neur2D cells, have opposing effects on structural plasticity, either facilitating (GFRα1a) or inhibiting (GFRα1b) neurite outgrowth.[35,43] Although our investigation did not extend to a detailed characterization of the effects of GFRα1a knockdown on structural plasticity in NPCs, our correlational data from postmortem human brain suggest a similar dissociation between GFRα1 isoforms insofar as they relate to another measure of neuroplasticity. Among psychiatrically healthy individuals, both GFRα1a and b correlated positively with one another, as well as with BLA expression of the neuroplastic protein DCX. Conversely, depression was associated with a complete disruption of GFRA1 isoform co-expression and a

strongly negative relationship between BR1-containing GFRA1 mRNAs and DCX levels. Whether this association reflects a direct, causal relationship between changes in GDNF signalling and DCX expression remains to be seen. Regardless, identifying the precise effects of decreased GFRα1a to b ratio on neuronal gene expression will be crucial to understanding the ramifications of the changes reported here.

Careful consideration must also be given to how changes in cells' interpretation of GDNF signalling may interact with changes in GDNF levels themselves. The efficacy of many antidepressants appears to rely, at least in part, on their ability to enhance neuroplasticity, and many achieve this by upregulating expression of neurotrophins.[44,45] In doing so, antidepressant treatment may overcome the altered GFRα1a:GFRα1b ratio by increasing GDNF levels to the point that receptor competition is sufficiently reduced to normalize the relative amount of GFRα1a to b signalling. To completely reverse the effects of reduced GFRα1a expression, however, this approach would likely require increasing neurotrophin levels well beyond normal physiological levels; although we were able to reduce the differences in *fos* induction between miR-511 and control cells with a 50-fold increase in GDNF dosage, the groups remained statistically different. Long-term elevations in neurotrophin expression also have the potential to increase susceptibility to other maladaptive behavioural outcomes.[46] By contrast, when we replaced the soluble GFRα1 receptors of miR-511-transfected cells with those from control NPCs, MAPK activity was immediately and completely rescued without the need to increase GDNF levels. Elevating GFRα1a expression itself may therefore prove an alternate, and more efficient, means of normalizing GDNF signalling.

The present investigation has several limitations which will need to be addressed by future studies to clarify our findings. Human postmortem data is, by nature, correlational, and limited sample sizes combined with the large number of comparisons made during the course of this study necessitates confirmation by replication. As a result, the precise relationship between increased miR-511, decreased GFRα1a levels, and DCX expression in the human BLA remains unclear. Although the use of *in vitro* models can provide insight into the potential ramifications of reduced GFRα1a expression, the NPCs used here were less mature and more isolated than neurons in the adult human brain. They are therefore both unlikely to perfectly recapitulate changes in BLA neuron function, and unable to inform us on the effects of GFRα1a on other cell populations (for example, astrocytes, which express the GFRα1 co-receptor RET, represent a principal source of GDNF and may be affected by altered soluble GFRα1 levels). Finally, although it is unlikely that miR-511 would have a comparable effect in rodent models due to species differences in the composition of the GFRA1 3'-UTR (which does not appear to vary between GFRα1a- and b-coding isoforms in mice), animal models may nonetheless be helpful in assessing how reduced neuronal GDNF/soluble GFRα1a/NCAM signalling affect behaviour.

In conclusion, the present study reveals depression-related changes in central GDNF signalling. In the BLA, these appear to be mediated by microRNAs, including miR-511, which can qualitatively alter neuronal responses to GDNF by regulating GFRα1a expression independently of GFRα1b. Altered GFRα1a to GFRα1b ratio is associated with greatly enhanced MAPK signalling and, in the human brain, with a reversal of the relationship between GFRα1 and DCX expression. Taken together, our data support a role for GDNF in depression, and highlight the importance of isoform-specific GFRα1 receptor regulation in shaping neuronal responses to this neurotrophin.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to the next of kin of the deceased for their support and for the donation of brain tissue to the Douglas-Bell Canada Brain Bank. We thank the Quebec coroner's office as well as the expert staff of the Douglas-Bell Canada Brain Bank (Maâmar Bouchouka, Josée Prud'homme, Danielle Cécyre, Kirsten Humbert and Lucie Ratelle). We are grateful to Carl Ernst for his input and advice, as well as for providing the human NPCs used in all *in vitro* experiments. This work was supported by operating grants from the Canadian Institutes of Health Research (NM: CIHR grant number MOP-111022), the American Foundation for Suicide Prevention (Pilot grant to NM), and an infrastructure grant from the Canada Foundation for Innovation (NM: CFI grant number 16309). MM is the recipient of a CIHR doctoral award, and NM is the Bell Senior Fellow in Mental Health, as well as CIHR New Investigator and FRQS chercheur-boursier.

AUTHOR CONTRIBUTIONS

MM was involved in the design and execution of this study, the optimization and execution of most experiments and the collection and analysis of most data. MAD contributed to the protein extraction of human brain samples and immunoblotting experiments. JPL contributed to the extraction of human RNA, qRT-PCR experiments and microRNA target prediction experiments and analyses. MicroRNA qRT-PCR experiments were planned and executed by JPL. LC was responsible for the maintenance of human NPCs and aided with NPC sample collection, and JPL helped optimize microRNA transfections. GT and JPL contributed to the design and interpretation of experiments. NM contributed to and coordinated the design and execution of all aspects of the study, including conception, data interpretation and analysis. The study was supported by NM, and the manuscript prepared by MM in consultation with all the authors.

REFERENCES

1 Vyas A., Mitra R., Rao BSS, Chattarji S. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* 2002; **22**: 6810–6818.
2 Padival MA, Blume SR, Rosenkranz JA. Repeated restraint stress exerts different impact on structure of neurons in the lateral and basal nuclei of the amygdala. *Neuroscience* 2013; **246**: 230–242.
3 Vyas A, Jadhav S, Chattarjo S. Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala. *Neuroscience* 2006; **143**: 387–393.
4 Maroun M, Ioannides PJ, Bergman KL, Kavushansky A, Holmes A, Wellman CL. Fear extinction deficits following acute stress associate with increased spine density and dendritic retraction in basolateral amygdala neurons. *Eur J Neurosci* 2013; **38**: 2611–2620.
5 Mitra R, Adamec R, Sapolsky R. Resilience against predator stress and dendritic morphology of amygdala neurons. *Behav Brain Res* 2009; **205**: 535–543.
6 Mitra R, Jadhav S, McEwen BS, Vyas A, Chattarji S. Stress duration modulates the spatiotemporal patterns of spine formation in basolateral amygdala. *Proc Natl Acad Sci USA* 2005; **102**: 9371–9376.
7 Mitra R, Ferguson D, Sapolsky RM. SK2 potassium channel overexpression in basolateral amygdala reduces anxiety, stress-induced corticosterone secretion and dendritic arborization. *Mol Psychiatry* 2009; **14**: 847–855.
8 Maheu ME, Davoli MA, Turecki G, Mechawar N. Amygdalar expression of proteins associated with neuroplasticity in major depression and suicide. *J Psychiatr Res* 2013; **47**: 384–390.
9 Duman RS, Monteggia LM. A neurotrophic model for stress-related mood disorders. *Biol Psychiatry* 2006; **59**: 1116–1127.
10 Pallavi P, Sagar R, Mehta M, Sharma S, Subramanium A, Shamshi F *et al.* Serum neurotrophic factors in adolescent depression: gender difference and correlation with clinical severity. *J Affect Disord* 2013; **150**: 415–423.
11 de Azevedo T, Mondin TC, Wiener CD, Marques MB, Fucolo Bde Á, Pinheiro RT *et al.* Neurotrophic factors, clinical features and gender differences in depression. *Neurochem Res* 2014; **39**: 1571–1578.
12 Otsuki K, Uchida S, Watanuki T, Wakabayashi Y, Fujimoto M, Matsubara T *et al.* Altered expression of neurotrophic factors in patients with major depression. *J Psychiatr Res* 2008; **42**: 1145–1153.
13 Tseng PT, Lee Y, Lin PY. Age-associated decrease in serum glial cell line-derived neurotrophic factor levels in patients with major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry* 2013; **40**: 334–339.
14 Diniz BS, Teixeira AL, Miranda AS, Talib LL, Gattaz WF, Forlenza OV *et al.* Circulating glial-derived neurotrophic factor is reduced in late-life depression. *J Psychiatr Res* 2012; **46**: 135–139.

15 Zhang X, Zhang Z, Xie C, Xi G, Zhou H, Zhang Y *et al.* Effect of treatment on serum glial cell line-derived neurotrophic factor in depressed patients. *Prog Neuropsychopharmacol Biol Psychiatr* 2008; **32**: 886–890.
16 Zhang X, Zhang Z, Sha W, Xie C, Xi G, Zhou H *et al.* Electroconvulsive therapy increases glial cell-line derived neurotrophic factor (GDNF) serum levels in patients with drug-resistant depression. *Psychiatry Res* 2009; **170**: 273–275.
17 Aquiar AS Jr, Stragier E, da Luz Scheffer D, Remor AP, Oliveira PA, Prediger RD *et al.* Effects of exercise on mitochondrial function, neuroplasticity and anxio-depressive behavior of mice. *Neuroscience* 2014; **271**: 56–63.
18 Liu Q, Zhu HY, Li B, Wang YQ, Yu J, Wu GC. Chronic clomipramine treatment restores hippocampal expression of glial cell line-derived neurotrophic factor in a rat model of depression. *J Affect Disord* 2012; **141**: 367–372.
19 Uchida S, Hara K, Kobayashi A, Otsuki K, Yamagata H, Hobara T *et al.* Epigenetic status of Gdnf in the ventral striatum determines susceptibility and adaptation to daily stressful events. *Neuron* 2011; **69**: 359–372.
20 Ma XC, Chen C, Zhu F, Jia W, Gao CG. Association of the GDNF gene with depression and heroin dependence, but not schizophrenia, in a Chinese population. *Psychiatry Res* 2013; **210**: 1296–1298.
21 Wang XC, Xu DJ, Chen GH, Xia Q, Liu LN. Association of two neurotrophic factor polymorphisms with efficacy of paroxetine in patients with major depressive disorder in a Chinese population. *Ther Drug Monit* 2014; **36**: 612–617.
22 Airaksinen MS, Saarma M. The GDNF family: signaling, biological functions and therapeutic value. *Nat Rev Neurosci* 2002; **3**: 383–394.
23 Paratcha G, Ledda F. GDNF and GFRalpha: a versatile molecular complex for developing neurons. *Trends Neurosci* 2008; **31**: 384–391.
24 Euteneuer S, Yang KH, Chavez E, Leichtle A, Loers G, Olshansky A *et al.* Glial cell line-derived neurotrophic factor (GDNF) induces neuritogenesis in the cochleat spiral ganglion via neural cell adhesion molecule (NCAM). *Mol Cell Neurosci* 2013; **54**: 30–43.
25 Ledda F, Paratcha G, Sandovai-Guzman T, Ibanez CF. GDNF and GFRalpha1 promote formation of neuronal synapses by ligand-induced cell adhesion. *Nat Neurosci* 2007; **10**: 293–300.
26 Paratcha G, Ibanez CF, Ledda F. GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream. *Mol Cell Neurosci* 2006; **31**: 505–514.
27 Lopez JP, Lim R, Cruceanu C, Crapper L, Fasano C, Labonte B *et al. M*iR-1202 is a primate-specific and brain enriched microRNA involved in major depression and antidepressant treatment. *Nat Med* 2014; **20**: 764–768.
28 Dweep H, Sticht C, Pandey P, Gretz N. MiRWalk–database: prediction of possible microRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform* 2011; **44**: 839–847.
29 Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res* **36**: D149–D153.
30 Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM *et al.* A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. *Cell* 2006; **126**: 1203–1217.
31 Krüger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res* 2006; **34**: W451–W454.
32 Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; **120**: 15–20.
33 Lopez JP, Fiori LM, Gross JA, Labonte B, Yerko V, Mechawar N *et al.* Regulatory role of miRNAs in polyamine gene expression in the prefrontal cortex of depressed suicide completers. *Int J Neuropsychopharmacol* 2014; **17**: 23–32.
34 Charlet-Berguerand N, Le Hir H, Incoronato M, di Porzio U, Yu Y, Jing S *et al.* Expression of GFRalpha1 receptor splicing variants with different biochemical properties is modulated during kidney development. *Cell Signal* 2004; **16**: 1425–1434.
35 Yoong LF, Wan G, Too H-PP. GDNF-induced cell signaling and neurite outgrowths are differentially mediated by GFRalpha1 isoforms. *Mol Cell Neurosci* 2009; **41**: 464–473.
36 Luzzati F, Bonfanti L, Fasolo A, Perretto P. DCX and PSA-NCAM expression identifies a population of neurons preferentially distributed in associative areas of different pallial derivatives and vertebrate species. *Cereb Cortex* 2009; **19**: 1028–1041.
37 Zhang XM, Cai Y, Chu Y, Chen EY, Feng JC, Luo XG *et al.* Doublecortin-expressing cells persist in the associative cerebral cortex and amygdala in aged nonhuman primates. *Front Neuroanat* 2009; **3**: 17.
38 Klempin F, Kronenberg G, Cheung G, Kettenmann H, Kempermann G. Properties of doublecortin-(DCX)-expressing cells in the piriform cortex compared to the neurogenic dentate gyrus of adult mice. *PLoS One* 2011; **6**: e25760.
39 Nielsen J, Gotfryd K, Li S, Kulahin N, Soroka V, Rasmussen KK *et al.* Role of glial cell line-derived neurotrophic factor (GDNF)-neural cell adhesion molecule (NCAM) interaction in the induction of neurite outgrowth and identification of a binding site for NCAM in the heel region of GDNF. *J Neurosci* 2009; **29**: 11360–11376.
40 Paratcha G, Ledda F, Ibanez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* 2003; **113**: 867–879.
41 Niethammer P, Delling M, Sytnyk V, Dityatev A, Fukami K, Schachner M *et al.* Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis. *J Cell Biol* 2002; **157**: 521–532.
42 Büttner B, Kannicht C, Reutter W, Horstkorte R. The neural cell adhesion molecule is associated with major components of the cytoskeleton. *Biochem Biophys Res Commun* 2003; **310**: 967–971.
43 Yoong LF, Pen ZN, Wan G, Too H-P. Tissue expression of alternatively spliced GFRα1, NCAM and RET isoforms and the distinct functional consequence of ligand-induced activation of GFRα1 isoforms. *Molec Brain Res* 2005; **139**: 1–12.
44 Duman RS. Role of neurotrophic factors in the etiology and treatment of mood disorders. *Neuromolecular Med* 2004; **5**: 11–25.
45 Castrén E, Rantamäki T. The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. *Dev Neurobiol* 2010; **70**: 289–297.
46 Chou D, Huang CC, Hsu KS. Brain-derived neurotrophic factor in the amygdala mediates susceptibility to fear conditioning. *Exp Neurol* 2014; **255**: 19–29.

Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)