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

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Supplementary Materials: Tables, Figures, and Methods

Supplementary Table 1. **Postmortem human brain sample information for protein, mRNA, and microRNA experiments**. Human BLA samples from psychiatrically-healthy controls (CTRL; n = 21) and depressed subjects having died by suicide (DS; n =16) were matched according to age, sex, postmortem interval (PMI), and brain pH, and did not differ significantly between groups. Group differences were assessed using two-tailed Student's *t*-tests (age, PMI, brain pH) and Fisher's exact test (Sex). Whenever possible protein, mRNA, and microRNA were collected for all subjects; group overlap is depicted by a Venn diagram.

PROTEIN

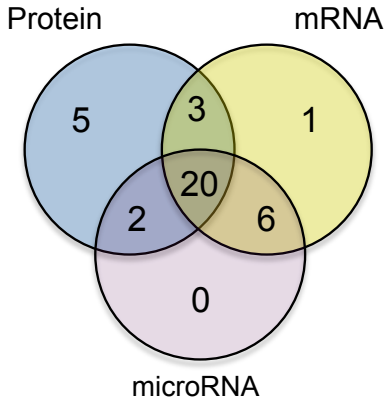
	CTRL	DS	p-value
N	18	12	N/A
Sex	15 M; 3 F	10 M; 2 F	1.000
Age (±SD)	44.56 (19.53)	41.33 (18.00)	0.652
PMI (±SD)	27.72 (14.50)	35.96 (23.55)	0.558
Brain pH (±SD)	6.51 (0.31)	6.58 (0.30)	0.245

mRNA

	CTRL	DS	p-value
N	16	14	N/A
Sex	13 M; 3 F	9 M; 5 F	0.417
Age (±SD)	45.9 (16.89)	41.21 (15.62)	0.442
PMI (±SD)	21.84 (14.08)	24.75 (24.55)	0.689
Brain pH (±SD)	6.52 (0.34)	6.59 (0.27)	0.348

microRNA

	CTRL	DS	p-value
N	15	13	N/A
Sex	11 M; 4 F	8 M; 5 F	0.689
Age (±SD)	50.53 (17.18)	41.69 (16.15)	0.175
PMI (±SD)	26.57 (16.98)	24.27 (25.49)	0.778
Brain pH (±SD)	6.52 (0.34)	6.59 (0.27)	0.514

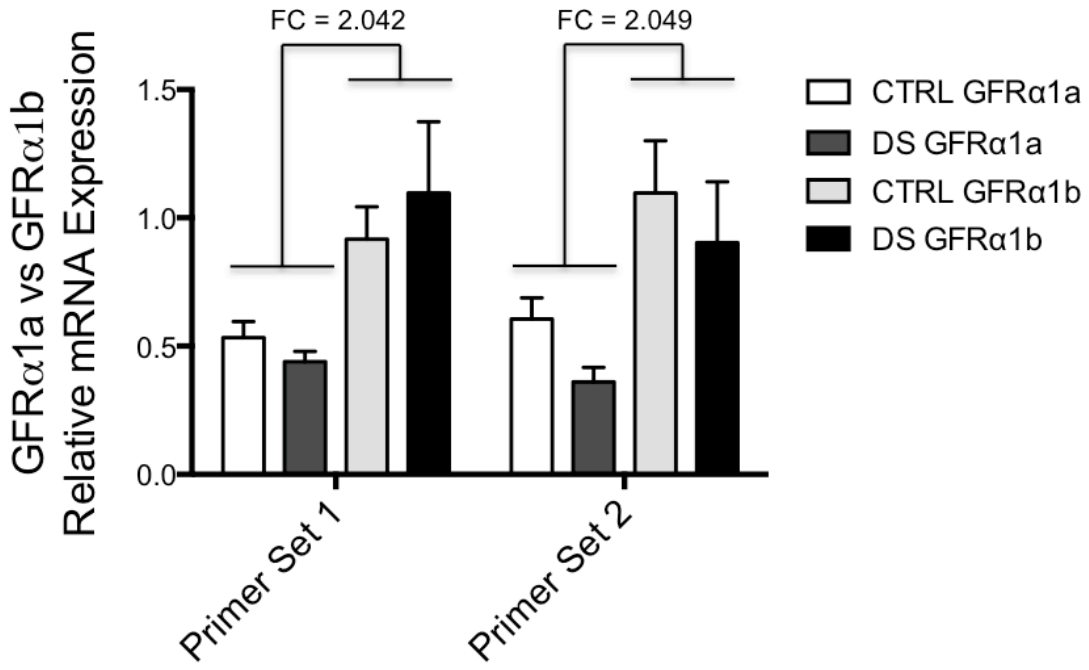


Supplementary Table 2. **Primers used for quantitative real-time PCR.**

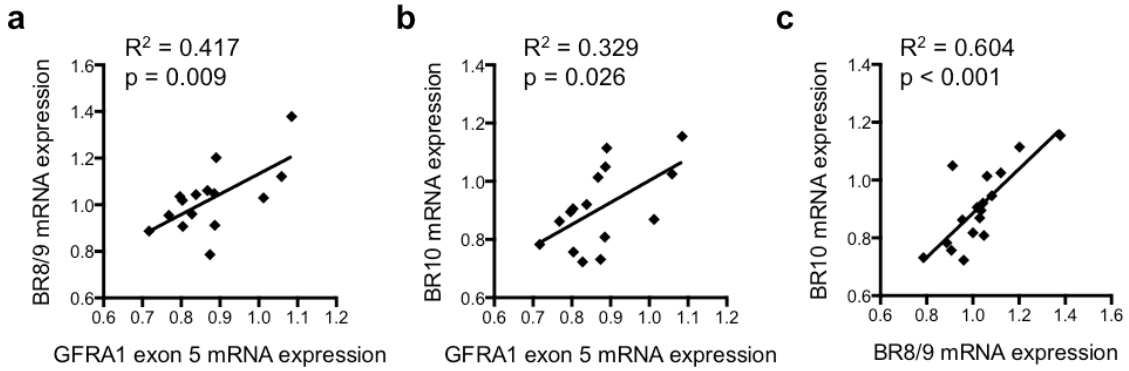
Gene target	Forward	Reverse
GAPDH	TTGTCAAGCTCATTTCCTGG	TGTGAGGAGGGGAGATTCAG
All GFRA1 transcripts	GTCGGGCAATACACACCTCT	TTGTGGTTATGTGGCTGGAA
Full length GFRA1 transcripts	GCTCTTGGACTTGCTCCTGT	GCTGAAGTTGGTCTCCTTGC
Truncated GFRA1 transcripts	ATTCCAGTGGAGCACATTCC	TTCTTGCAAATGTCGTCGAG
GFRa1a Exon 5	TCAGCAAGTGGAGCACATTC	TTCTTGCAAATGTCGTCGAG
miR-511 BR1	AGCTTCTTGTGATCCTTTGG	ATATCCCAAAGCCTTCTGAGT
miR-511 BR8/9	TTAGCTGTAGCTGAAGGCATTT	ATGCCCAATGACAGACCACAG
miR-511 BR10	TATCTGCTTTCCAGAGGACAC	GCTGTTGCAAAACAGCTATG
GFRa1a_Yoong et al. 2009	CATATCAGATGTTTTTCAGCAA	CAGACATCGTTGGACACG
GFRa1b_Yoong et al. 2009	TGGTCCCATTCATATCAGTGGA	CAGACATCGTTGGACACG
Fos	CCGGGGATAGCCTCTCTTAC	GTGGGAATGAAGTTGGCACT
Egr1	GACCGCAGAGTCTTTTCCTG	AGCGGCCAGTATAGGTGATG
Egr2	GGTGACCATCTTTCCCAATG	GGATATGGGAGATCCAACGA
NCAM Primer Set 1	TACACTTGGGAGGGGAACCA	GCCATCCCGAAACCATGAGA
NCAM Primer Set 2	GTACACTTGGGAGGGGAACC	GCAGAGGGGGTGTTGTAGAT
RET Primer Set 1	TGCCCAGTACCTACTCCCTC	TGGACGTTGATGCCACTGAA
RET Primer Set 2	TGGTTCTCAACCGGAACCTC	ACCGACACGTTGAAGTGGAG

Supplementary Table 3. **Antibodies used for immunoblotting.**

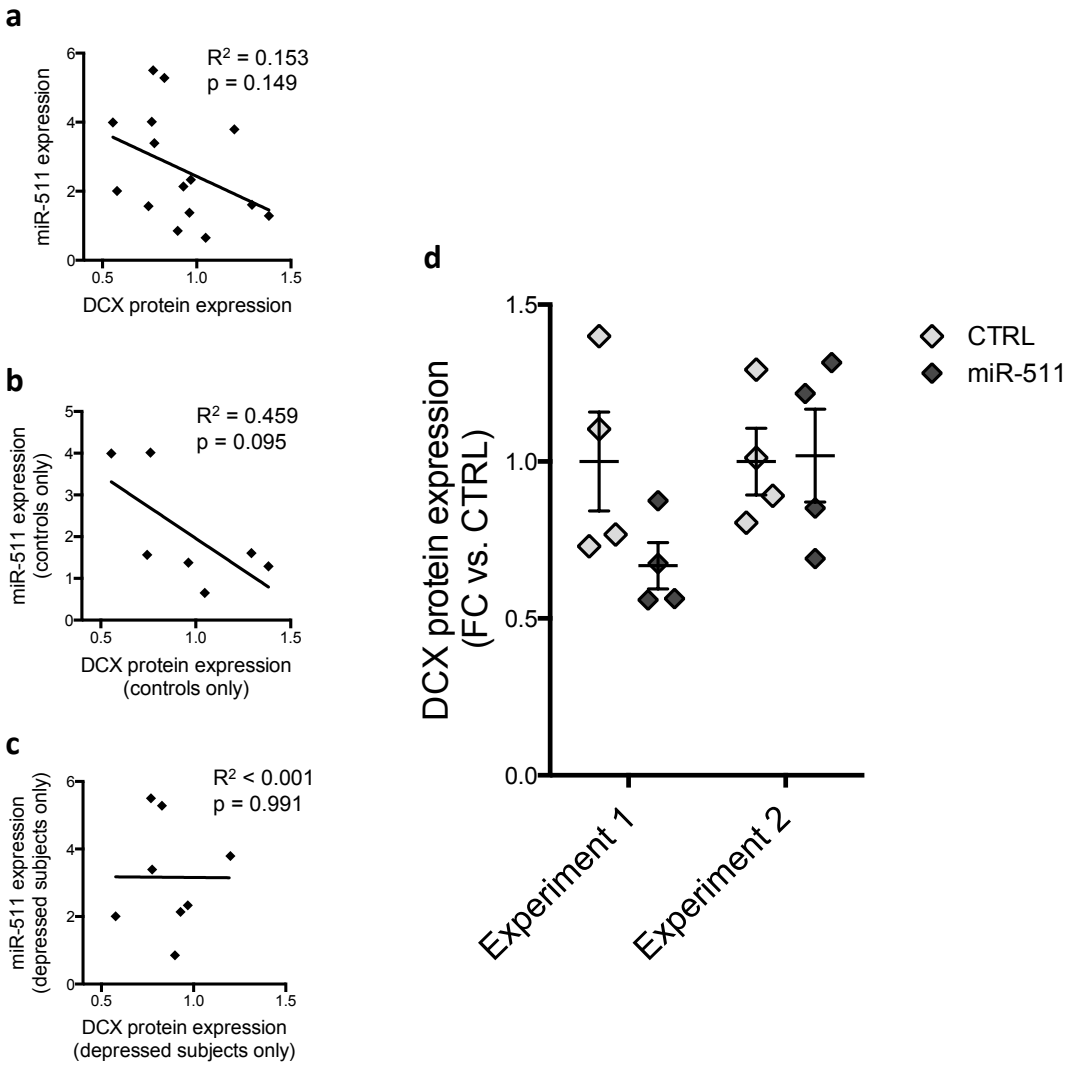
Protein Target	Manufacturer	Catalog #	Host Species	Concentration
Primary Antibodies				
Akt (pan)	Cell Signaling Technologies	4691	Rabbit	1:1000
DCX	Cell Signaling Technologies	4604	Rabbit	1:1000
GDNF	Santa Cruz	sc-328	Rabbit	1:200
GFRalpha1	Exalpha Biologics	X1138P	Rabbit	1:1000
NCAM1/CD56	Lifespan Biosciences	LS-C105629	Rabbit	1:1000
p44/42 MAPK (Erk1/2)	Cell Signaling Technologies	4695	Rabbit	1:1000
Phospho-Akt (Ser473)	Cell Signaling Technologies	4060	Rabbit	1:1000
Phospho-p44/42 MAPK (Erk1/2)	Cell Signaling Technologies	4370	Rabbit	1:1000
Ret	Abnova	H00005979-M07	Mouse	1:1000
Secondary Antibodies				
Actin-HRP	Santa Cruz	sc-1616	Goat	1:2000
Anti-mouse IgG-HRP	R & D Systems	HAF018	Donkey	1:2000
Anti-rabbit IgG-HRP	Amersham	NA934	Donkey	1:2000



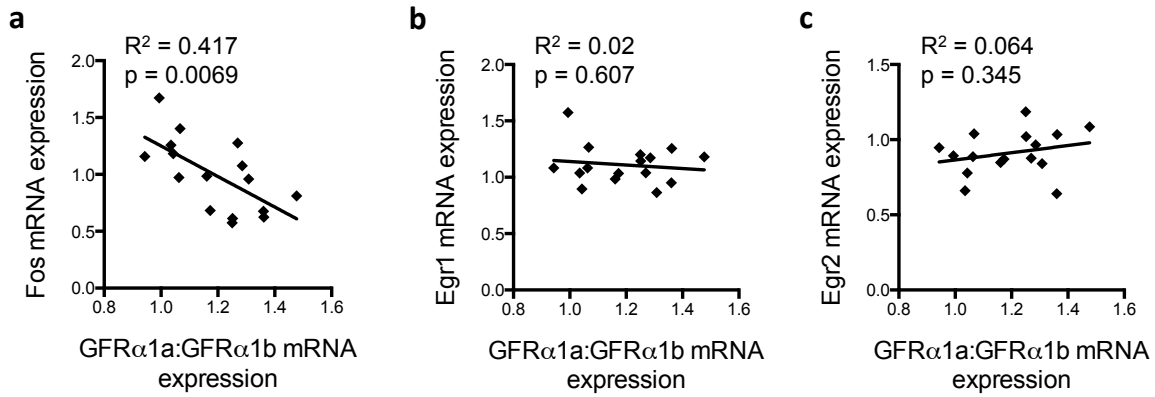
Supplementary Figure 1. **Relative expression of GFRα1a and GFRα1b in the human basolateral amygdala.** We assessed the relative expression of GFRα1a and b isoforms in control (CTRL; n = 14) and depressed subjects (DS; n = 13) using quantitative real-time PCR and two sets of primers. Primer Set 1 was previously validated by Yoong and colleagues[35]. For Primer Set 2, GFRα1a mRNA expression was assessed using primers designed to target GFRα1a exon 5 (see Supplementary Table 2). The absolute quantity of GFRα1a mRNA was then subtracted from the mRNA expression of all full-length GFRA1 transcripts in order to acquire an estimate of GFRα1b expression. Primer Set 1 and Primer Set 2 yielded remarkably similar results, providing GFRα1a to GFRα1b fold change estimates of 2.042 and 2.049, respectively. Among controls, the ratio of GFRα1a to GFRα1b variants is approximately 1 to 1.9; among depressed subjects, the ratio is lower (1 to 2.6). Thus, in the human BLA GFRα1b mRNA expression is roughly 2-fold higher than GFRα1a expression.



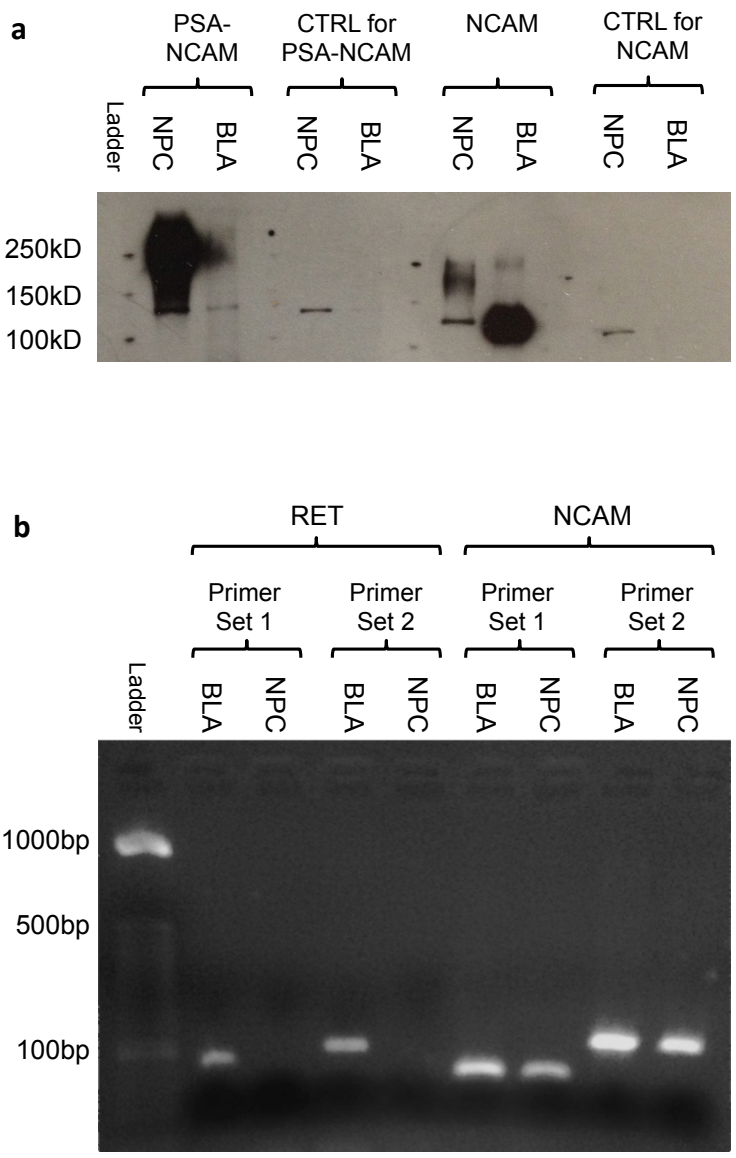
Supplementary Figure 2. **GFRA1 mRNA correlations in human NPCs.** Correlations between (**a**) expression of GFRα1a-specific exon 5 and BR8/9-containing GFRA1-L mRNA, (**b**) GFRα1a-specific exon 5 and BR10-containing GFRA1-L mRNA, and (**c**) BR8/9- and BR10-containing GFRA1-L mRNA. For qRT-PCRs, each sample was run in quadruplicate, and all Pearson correlations are pairwise.



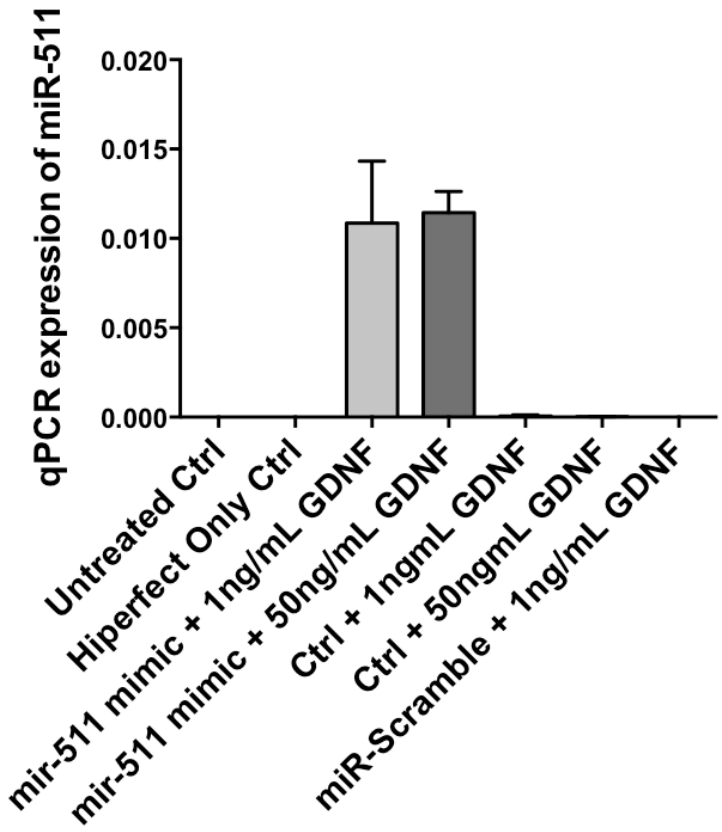
Supplementary Figure 3. **Relationship between miR-511 and the neuroplastic marker doublecortin (DCX) in the human BLA.** Correlations between miR-511 and DCX protein expression in (**a**) all subjects, (**b**) controls (CTRL) only, and (**c**) depressed subjects (DS) only. The relationship between DCX protein levels and GFRα1 expression does not appear to be mediated directly by miR-511, nor was (**f**) transfection of human NPCs with miR-511 sufficient to alter DCX protein levels. Immunoblotting data were confirmed by independent technical replication. For qRT-PCRs, each sample was run in quadruplicate. All Pearson correlations are pairwise, and analysis of group comparisons were completed using Student's two-tailed t-tests.



Supplementary Figure 4. **Immediate early gene signaling in miR-511-transfected human NPCs.** (**a**) The ratio of GFRα1a to GFRα1b mRNA correlated negatively and significantly with Fos expression following GDNF administration, suggesting that altered Fos activity in miR-511-transfected cells is related to GFRα1a levels. Conversely, (**b**) Egr1 and (**c**) Egr2 mRNA expression following treatment with GDNF did not appear to be related to GFRα1a expression. For qRT-PCRs, each sample was run in quadruplicate. All Pearson correlations are pairwise, and analysis of all group comparisons were completed using Student's two-tailed t-tests.



Supplementary Figure 5. **Expression of GDNF/GFRα1 co-receptors NCAM and RET in human BLA and NPCs.** (**a**) Immunoblotting of pooled BLA ($n > 6$) and pooled untreated NPCs ($n = 3$) revealed positive expression of both polysialylated and unsialylated NCAM protein in human BLA and NPCs at 140kD and 180kD (isoforms capable of mediating GDNF signaling). (**b**) PCR using pooled BLA (n > 6) and pooled untreated NPCs (n = 6) as well as 2 sets of primers capable of amplifying all transcript variants of both RET and NCAM (see Supplementary Table 2) revealed robust expression of NCAM in human NPCs, but no detectable RET. Immunoblotting and PCR results were confirmed by independent technical replication.



Supplementary Figure 6. **Quantitative real-time PCR expression of miR-511 in human NPCs.** qRT-PCRs with human NPCs (n = 3 per group, with each sample run in quadruplicate) revealed no endogenous expression of miR-511 in untransfected cells.

Supplementary Materials: Methods

Human brain samples

For all subjects, cause of death was determined by the Quebec Coroner's Office, and psychological autopsies completed as previously described[47]. Briefly, the Structured Clinical Interview for DSM-IV Psychiatric Disorders (SCID-I) was completed by a trained interviewer with one or more informants best acquainted with the deceased, and the results assessed by a panel of clinicians in conjunction with Coroner's notes, medical records, and case reports in order to obtain a consensus diagnosis. Age, postmortem interval, and brain pH did not differ between groups.

Isolation and processing of microRNA, mRNA, and protein

Total RNA was isolated from frozen brain samples using either the Lipid Tissue Mini Kit (mRNA only) or the miRNeasy Mini Kit (mRNA and microRNA) according to the manufacturer's protocol (Qiagen, Toronto, ON, Canada). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and only mRNA samples with a RIN value ≥ 5 were included in this study. Reverse transcription of mRNA was completed using M-MLV reverse transcriptase and oligo(dT)16 primers (Life Technologies, Burlington, ON, Canada). TaqMan RT-PCR microRNA assays (Applied Biosystems, Burlington, ON, Canada) were used for microRNA reverse transcription.

For protein extractions, tissues were lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO) and homogenized by sonication. Total protein content was assessed using the BCA Protein Assay kit (Pierce, Rochford, IL).

Immunoblotting

Protein samples were resolved through SDS-PAGE and transferred nitrocellulose membranes. Membranes were blocked at room temperature, then incubated in a solution containing primary antibody, followed by incubation in a solution containing HRP-conjugated secondary antibody (see Supplementary Table 3 for a complete list of

antibodies used). Signals were revealed with ECL against film and the optical density of target bands assessed using the MCID system (InterFocus Imaging Ltd, Cambridge, UK) and ImageJ software (National Institutes of Health, Bethesda, MD). Specificity of the signal was confirmed with the use of molecular weight markers and control conditions in which the primary antibody was omitted. Protein expression of samples was normalized using β-actin, and interblot comparisons normalized using pooled calibrators run in triplicate on every gel.

Immunohistochemistry

Three uncharacterized fresh-fixed BLA samples were provided by the Suicide section of the Douglas-Bell Canada Brain Bank for qualitative immunohistochemistry. Fresh human BLA samples used for immunohistochemistry were briefly fixed in 10% formalin then incubated for 24 hours each in 15% and 30% sucrose prior to being flash-frozen in cold isopentane and sectioned at 50μm on a freezing microtome. Fresh-fixed BLA sections were rinsed in PBS, and incubated in 0.9% H_2O_2 for 10 minutes prior to antigen retrieval with 0.1M Tris-HCl (pH 8.22). Samples were then blocked in PBS containing 3% normal serum and incubated in anti-GFRa1 (1:100; Exalpha Biologicals, Shirley, MA), anti-RET (1:500; Abnova, Jhongli City, Taiwan), or anti-NCAM1 (1:1000; Lifespan Biosciences, Seattle, WA) for 72 hours at 4ºC. Afterwards, BLA samples were rinsed and incubated in a biotinylated secondary antibody solution (1:200; Vector Laboratories, Burlingame, CA) for 4 hours at room temperature prior to signal amplification with the Vectastain Elite ABC kit and development with DAB (Vector Laboratories, Burlingame, CA). Sections were mounted on slides, dehydrated, cleared with xylenes, and coverslipped with permount. Images were captured using a Leica DM 2500 mounted with a DFC420 camera and 10x (NA 0.3) and 40x (NA 0.75) objectives.

Human neural progenitor cell (NPC) culture

Human neural progenitor cells (NPCs) derived from the 8330-8 induced pluripotent stem cell line[48] were provided by C. Ernst at McGill University. Culture plates were coated with 200 μg mL^{-1} of poly-l-ornithine hydrobromide (Sigma-Aldrich, St. Louis, MO) and 5 mg mL^{-1} laminin (Sigma-Aldrich, St. Louis, MO). NPCs were maintained in a

humidified incubator at 37ºC and a CO_2 concentration of 5%, and grown in 70% DMEM (Invitrogen, Burlington, ON, Canada) and 30% Hams F12 (Mediatech, Laval, QC, Canada) medium containing penicillin-streptomycin (Invitrogen, Burlington, ON, Canada), B-27 supplement (Invitrogen, Burlington, ON, Canada), and 20n g mL^{-1} each of human EGF (Sigma-Aldrich, St. Louis, MO) and FGF (R&D Systems, Burlington, ON, Canada). Once cells achieved ~90% confluence, neuronal differentiation was induced by removing EGF and FGF from the cell medium.

Human NPC transfections

Two weeks after induction of differentiation, NPCs were transfected using HiPerFect transfection reagent alone, or in conjunction with 10nM miScript miR-511 Mimic or 10nM AllStars Negative Control SiRNA (Qiagen, Toronto, ON, Canada). Transfections were completed according to the manufacturer's protocol, and over-expression of miR-511 assessed at 24hrs using qRT-PCR (Supplementary Fig 8). For gene expression studies, NPCs were collected 48hrs after transfection and processed for RNA extraction. For studies examining GDNF-induced immediate early gene (IEG) signaling, cells were collected 72 hours after transfection. For protein expression experiments and Akt/MAPK GDNF signaling studies, NPCs were re-transfected a second time 3.5 days after the initial treatment for a total of 1 week of miR-511 over-expression.

Since HiPerFect-only controls did not differ from AllStars Negative Control SiRNA-transfected cells in any measure examined, all analyses compared miR-511 mimic-transfected cells (miR-511) with AllStars Negative Control SiRNA-transfected cells (CTRL).

References

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48 Sheridan SD, Theriault KM, Reis SA, Zhou F, Madison JM, Daheron L *et al*. Epigenetic characterization of the FMR1 gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile X syndrome. PLoS One 2011; 6: e26203.