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Author manuscript

*J Neurochem.* Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

*J Neurochem.* 2016 October ; 139(2): 197–207. doi:10.1111/jnc.13761.

## MicroRNAs 29b and 181a down-regulate the expression of the norepinephrine transporter and glucocorticoid receptors in PC12 cells

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### Abstract

MicroRNAs are short non-coding RNAs that provide global regulation of gene expression at the post-transcriptional level. Such regulation has been found to play a role in stress-induced epigenetic responses in the brain. The norepinephrine transporter (NET) and glucocorticoid receptors are closely related to the homeostatic integration and regulation after stress. Our previous studies demonstrated that NET mRNA and protein levels in rats are regulated by chronic stress and by administration of corticosterone, which is mediated through glucocorticoid receptors. Whether miRNAs are intermediaries in the regulation of these proteins remains to be elucidated. The present study was undertaken to determine possible regulatory effects of miRNAs on the expression of NET and glucocorticoid receptors in the noradrenergic neuronal cell line. Using computational target prediction, we identified several candidate miRNAs potentially targeting NET and glucocorticoid receptors. Western blot results showed that overexpression of miR-181a and miR-29b significantly repressed protein levels of NET, which is accompanied by a reduced [<sup>3</sup>H] NE uptake, and glucocorticoid receptors in PC12 cells. Luciferase reporter assays verified that both miR-181a and miR-29b bind the 3'UTR of mRNA of NET and glucocorticoid receptors. Furthermore, exposure of PC12 cells to corticosterone markedly reduced the endogenous levels of miR-29b, which was not reversed by the application of glucocorticoid receptor antagonist

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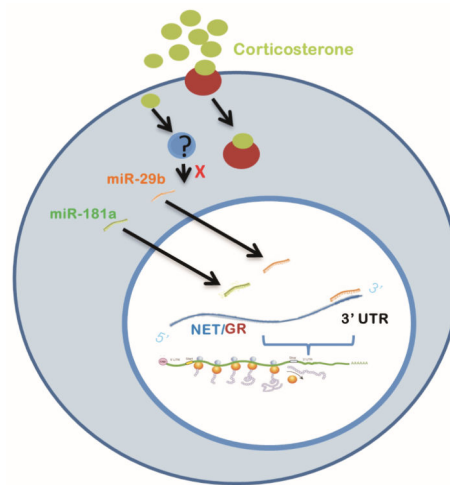
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mifepristone. These observations indicate that miR-181a and miR-29b can function as the negative regulators of NET and glucocorticoid receptor translation *in vitro*. This regulatory effect may be related to stress-induced upregulation of the noradrenergic phenotype, a phenomenon observed in stress models and depressive patients.

## Graphical abstract

This study demonstrated that miR-29b and miR-181a, two short-non-coding RNAs that provide global regulation of gene expression, markedly repressed protein levels of norepinephrine (NE) transporter and glucocorticoid receptor (GR), as well as NE uptake by binding the 3' UTR of their mRNAs in PC12 cells. Also, exposure of cells to corticosterone significantly reduced miR-29b levels through a GR-independent way.



## Keywords

microRNA; stress; norepinephrine transporter; glucocorticoid receptor; gene regulation; PC12 cells

## Introduction

MicroRNAs (miRNAs) are generally around 22-nucleotide-long noncoding RNAs that play an important role in gene-regulation (Makeyev & Maniatis 2008). They function by binding to complementary sequences on 3' UTR regions of target mRNA molecules and to repress translation and silence gene expression (Yelamanchili *et al.* 2010). By this post translational regulation, miRNAs are involved in a plethora of processes, such as cell proliferation (Brennecke *et al.* 2003), apoptosis, differentiation, development (He & Hannon 2004), and basic cellular pathways (Mendell 2005). The human genome contains more than 500 miRNAs and each individual miRNA can usually interact with a few hundred of target mRNAs (Schratt 2009, Broderick & Zamore 2011). It is well documented that around 70% of miRNAs are expressed in brain, involved in many crucial processes in central nervous system and play crucial role in dendritic spine formation, neurite growth (Esteller 2011), neuronal development, neuronal differentiation (Yelamanchili *et al.* 2010), neuronal

plasticity, apoptosis (Kosik 2006), dendritic arborization, and synapse formation and maturation (Schratt 2009). While it has been well known that miRNAs are essential to maintain the correct functioning mechanism in the nervous system (Esteller 2011), their dysregulation has been associated with several neurodegenerative disorders including Parkinson's disease, Alzheimer's diseases, Huntington disease (Saugstad 2010, Yelamanchili et al. 2010, Broderick & Zamore 2011, Esteller 2011), and a variety of psychiatric diseases such as major depression, schizophrenia and mood disorders (Beveridge et al. 2010, Dwivedi 2011b, O'Connor et al. 2012, Hansen & Obrietan 2013). Thus far, their involvement in these diseases has made it important to investigate them as the regulatory and therapeutic molecules (Wahid et al. 2010, Dwivedi 2011a). However, such kinds of studies are only beginning to emerge, in which miRNAs' involvement in the regulation of the noradrenergic phenotypes is almost absent.

The norepinephrine (NE) transporter (NET), is a member of the Na/Cl-dependent monoamine transporter family and selectively located on the presynaptic terminals of noradrenergic neurons (Dziedzicka-Wasylewska et al. 2006). It plays a crucial role in maintaining the presynaptic and postsynaptic NE homeostasis by re-uptaking more than 90% of released NE back to presynaptic terminals (Axelrod & Kopin 1969, Amara & Kuhar 1993). Therefore, changes in NET expression would significantly influence NE levels in the synapses which will in turn highly affect noradrenergic transmission. Also, the NET is one of the main transporters serving as a target of antidepressant drugs by enhancing noradrenergic transmission (Dziedzicka-Wasylewska et al. 2006). Given NET is one of the key proteins to regulate noradrenergic transmission and its involvement in the action of certain antidepressants, abnormal NET expression and function could contribute to the development and treatment of depression. For example, NET knockout mice display significantly less depressive-like behaviors than wild type controls (Xu et al. 2000), and are more aggressive in early phases of stress and demonstrate inhibition of depressive-like behavior in chronic stress models (Haller et al. 2002). These findings suggest that depressive behavior requires functional NET (Haller et al. 2002, Haenisch et al. 2009). Hence, the regulation of NET seems to be important for the etiological exploration and therapeutic strategy of major depression. However, such regulatory studies are limited. On the other hand, as the important mediators of glucocorticoids released during stress, corticosteroid receptors play a critical role in axis and in mediating brain functions. As such, an impaired corticosteroid receptor signaling has been hypothesized as a key mechanism in the pathogenesis of depression (Holsboer 2000). Of the two types of corticosteroid receptors, is only modestly occupied during normal physiological conditions and needs higher glucocorticoid concentrations to be fully activated in the state of stress. For this reason, the GR is considered to be important in depression (De Kloet et al. 1998). Regulations of GR expression, nuclear translocation, and GR-mediated gene transcription have become an important line of research regarding the molecular mechanisms underlying development and therapy of depression.

Our previous study demonstrated that chronic social defeat significantly increased expression levels of NET in the rat brain (Chen et al. 2012). This observation is consistent with *in vitro* study, in which stress-relevant doses of corticosterone and corticotropin-releasing factor significantly increased mRNA and protein levels of NET, as well as the

uptake of [<sup>3</sup>H]NE in SK-N-BE(2)C cells and PC12 cells (Sun *et al.* 2010). Interestingly, both stress- and corticosterone-induced NET upregulations are mediated through corticosteroid receptors (Sun *et al.* 2010, Chen *et al.* 2012). However, the results for the regulatory effects of stress on GR expression in the brain have been controversial. Our previous experiments showed that chronic stress increased GR expression in the locus coeruleus and raphe nucleus (unpublished data), a finding consistent with one previous report (Li *et al.* 2011), and exposure of cells to dexamethasone markedly increased total GR and nuclear GR levels (Zha *et al.* 2011). On the contrary, other laboratories reported no significant alteration of, or a reduced GR expression in the locus coeruleus (LC) after single-prolonged stress (Sabban *et al.* 2015), chronic stress (Makino *et al.* 2002) or chronic exposure to corticosterone (Wang *et al.* 2015), indicating stress-induced GR alterations in the LC are complex, though GR levels are very pronounced in the noradrenergic cells of the LC (Sabban *et al.* 2015). Clearly, more investigations are required. Nevertheless, it is possible that there is a functional linkage between noradrenergic phenotypes and GR during chronic stress, as noradrenergic neurons in the LC can be a target of glucocorticoids through GR (Markey *et al.* 1982, Wang *et al.* 1998). Given that chronic stress precipitates depression (Hammen 1991) and dysregulation of the noradrenergic phenotypes and GR are related to the development of depression, clarification of the molecular mechanisms underlying their regulation is an important step to be pursued, facilitating the search for effective therapies for people with major depression and other stress-related diseases. In the present study, using a combination of in silico prediction of miRNAs binding sites, miRNAs overexpression, constructs of the 3'UTR of NET and GR genes, as well as endogenous expression investigation of miRNAs, we identified miR-181a and miR-29b act as the regulator of NET and GR.

## Materials and Methods

### Cell culture, miRNA transfection and drug exposure

PC12 cells (ATCC, CRL-1721, Manassas, VA, USA) were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, 10% horse serum, penicillin (final concentration, 25 U/ml) and streptomycin (final concentration 25 µg/ml), at 37°C in humidified air containing 5% CO<sub>2</sub>. Culture medium and supplements were obtained from the Life Technologies Corporation, ThermoFisher Scientific (Grand Island, NY). The subcultured cells, when they reached to 70-85% confluence, were transfected with miRNA mimics, mimic control, or negative control (40nM, Life Technologies, Carlsbad, CA) by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Then the cells were incubated with the transfection complexes under normal growth conditions. Forty-eight hours after transfection, cells were harvested after washing twice with fresh, ice-cold phosphate buffer solution (PBS) and immediately lysed for isolation of RNA or protein measurements. In the separate experiments, PC12 cells were treated with 100 nM corticosterone, or 100 nM corticosterone plus 3 µM mifepristone, for 7 days. miRNAs were measured by real-time PCR after harvest. All experiments were conducted in compliance with the ARRIVE guidelines.

### Construct of luciferase reporter gene plasmid and luciferase reporter assays

To confirm that the validated miRNAs target the 3'-UTR of NET, the 3'-UTR segment of NET gene was amplified by PCR from PC12 genomic DNA and cloned to the downstream of red firefly gene of pmirGlo vector. The NET 3'-UTR-bearing pmirGlo and validated miRNAs were co-transfected into PC12 cells with Lipofactamine 2000. At 48 h after transfection, cells were lysed, and luciferase reporter activity was assayed. Briefly, after washing three times with PBS, cells were lysed in 100  $\mu$ l of 100 mM KPO<sub>4</sub> buffer (pH 7.8), which contained 1 mM dithiothreitol and 0.5% (vol/vol) Triton X-100. Activity assays were performed by measurement of luminescence in a Biocounter M2500 luminometer (Lumac, Landgraaf, The Netherlands) for 20 sec immediately after addition of luciferin assay buffer (100 mM Tris acetate (pH 7.8), 10 mM MgOAc, 100 mM EDTA, 2 mM ATP (pH 7.0) and 74  $\mu$ M luciferin). Luciferase activities were normalized to  $\beta$ -gal activity to correct for variations in transfection efficiencies. Luciferase signals of red firefly and green *Renilla* were measured by GloMax Microplate Multimode Reader.

### Detection of NET protein levels

Protein levels of NET and GR were measured by western blotting as reported before (Sun et al. 2010). Briefly, after twice washing with ice-cold PBS, cells were lysed with lysis buffer (50 mM Tris pH7.6, 150 mM NaCl, 2 mM EDTA, 1% NP40 and SigmaFast Protease Inhibitors) and collected. The total protein concentration in the supernatant of cell preparations was measured with Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Fifty micrograms of protein from each sample were loaded onto 10% sodium lauryl sulfate-polyacrylamide gels, followed by electrophoresis. Then, proteins were transferred on Amershan Hybond-ECL membrane (GE Healthcare, NJ), which were blocked in 5% milk in Tris-buffered saline with Tween 20 (TBST) buffer (25 mM Tris, 140 mM NaCl, 3 mM KCl, pH 7.4; and 0.1% Tween 20 v/v was added before use) for 1 h at room temperature. These membranes were further incubated with primary antibodies (polyclonal antibodies against NET from rabbit, 1:330 dilution; Alpha Diagnostic Intl. Inc, San Antonio, Texas; polyclonal antibody against GR: 1:500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for overnight at 4°C. On the next days, after 3 time washing with TBST buffer, these membranes were incubated with 2nd antibodies (horseradish peroxidase-conjugated anti-rabbit IgG, 1:3000; Amersham Biosciences, Little Chalfont, UK) at room temperature. Finally, membranes were treated with Enhanced Chemiluminescence Western Blotting Detection Reagents and exposed to Kodak BioMax MR Films (GE Healthcare, Marlborough, MA). The imaging software (Molecular Dynamics IQ solutions, Molecular Dynamics, Inc., Sunnyvale, CA) was used to quantify the density of bands. The  $\beta$ -actin as the loading control was measured by the same procedure after stripping of the membrane and incubating it with mouse anti- $\beta$ -actin antibody. As usual, linear standard curve was generated from optical densities (ODs) of bands by a dilution series of total proteins prepared from cells. OD values of NET and GR signals were normalized by  $\beta$ -actin immunoreactivities to assess equal protein loading. Normalized values were then averaged for all replicated gels and used to calculate the relative changes of the same gel. Also, the specificity of antibodies used in these experiments was tested when each new lot of the antibody was ordered.

## RNA isolation and Taqman-based quantitative real-time polymerase chain reaction (qPCR) analysis for miRNA

Total RNA was extracted using RNeasy lysis reagent (Qiagen, Crawfordsville, IN, USA), and was converted into cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems/Life technologies, Foster City, CA) according to the manufacturer's protocol. Real-time PCR was conducted using a Taqman® microRNA assay kit (Applied Biosystems/Life technologies, Grand Island, NY). The 20 µl PCR included 1 µl Taqman MicroRNA assay (20X), 1.33 µl RT product, 10 µl Taqman 2× Universal PCR Master mix, No AmpErase UNG<sup>a</sup>, and 7.67 µl Nuclease-free water. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of miR-181a/miR-29b was normalized using U6 as the internal control. Predesigned primer/probes (Applied Biosystems) for miRNAs and rat U6 were purchased from Applied Biosystems. Measurements were normalized to U6 (Ct) and comparisons calculated as the inverse log of  $2^{-\Delta\Delta Ct}$  to give the relative fold change in respective miRNAs/U6 snRNA levels (Livak & Schmittgen 2001). All reactions were run in triplicate, each using separate sets of samples.

## Uptake of [<sup>3</sup>H]NE

[<sup>3</sup>H]NE uptake in PC12 cells was carried out using the similar procedure as described previously (Zhu & Ordway 1997). Briefly, PC12 cells treated with miRNAs 48 hours ago were washed twice with 4 ml Krebs-Ringer-HEPES buffer (KRH; in mM: NaCl 130, KCl 1.3, CaCl<sub>2</sub> 2.2, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, HEPES 10, D-glucose 10, ascorbic acid 0.1 and pargyline 0.1; pH adjusted to 7.4 with HCl). Cells were then pre-incubated in 1.5 ml warm KRH buffer at 37°C for 5 min. Ninety nM [<sup>3</sup>H]NE (Perkin-Elmer Life Science, Boston, MA) were added and incubated for 5 min at 37°C to initiate uptake assays, which was removed by aspiration of incubation solutions followed by 2 rapid washes with ice-cold KRH buffer. Cells were then lysed with 1 ml 0.1% v/v Triton X-100 (in 5 mM Tris HCl, pH 7.4), and 0.5 ml of the lysate was used to measure the radioactivity in a liquid scintillation counter (Beckman LS3801, Irvine, CA, USA). The specific uptake of [<sup>3</sup>H] NE by the NET was defined as the difference between uptake in the absence of and in the presence of 100 µM desipramine. Protein concentrations in cell lysates were determined by the BCA Protein Assay.

## Statistics

All data were from separate experiments. The number of replicates is showed in the figure legend. The paired Student's *t*-test assay is used for the data which had only 2 groups in a comparison, such as those in Fig. 6. The analysis of variance (ANOVA) and *post hoc* Newman-Keuls tests were used for analysis of data when multiple treatment groups were compared. Differences between groups or treatments were recognized as statistically significant at  $p < 0.05$ .

## Results

### ***In silico* prediction of candidate miRNAs targeting NET and GRs**

Three leading miRNA target prediction algorithms: miRanda (<http://microrna.sanger.ac.uk/sequences/>) (John *et al.* 2004), PicTar (<http://pictar.mdc-berlin.de/>) (Krek *et al.* 2005), TargetScan (<http://www.targetscan.org/>) (Grimson *et al.* 2007), are used to identify miRNAs affecting NET and GR activities via binding to their 3'UTR site. To determine genes that were similarly identified by two or more of these algorithms, the online program Matchminer (<http://discover.nci.nih.gov/matchminer/index.jsp>) (Bussey *et al.* 2003) was also used. By these computational target prediction analyses, we identified several potential miRNAs that have complementarity to the 3'-untranslated region (UTR) of the mRNAs of NET (Table 1) and GRs (Table 2) for further experimental testing. Interestingly, the UTRs of both NET and GR genes harbor the putative binding sites for miR-29, miR-181a, miR-9 and miR-124.

### **MiR-181a and miR-29b down-regulate proteins of NET and GRs in PC12 cells**

MiRNAs show high ability to bind to the 3'UTR of mRNAs of target genes; thereby they repress their translation and ultimately lead to reduction of protein levels. Therefore, all mimics of the miRNAs targeting the 3'UTR of NET and GRs, as well as the mimic control and negative control were transfected into PC12 cells. Forty eight hours after transfection, cells were harvested. Protein levels of NET and GRs were assayed by western blotting. As shown in Figs. 1 and 2, the overexpression of miR-181a and miR-29b dramatically downregulated NET ( $F_{3, 19}=4.75$ ,  $p<0.05$ ) and GR ( $F_{3, 19}=4.36$ ,  $p<0.05$ ) protein levels, as compared to those of the mimic control and negative control. In contrast, transfection with respective miR-9, miR-124, miR-146a, miR-377, miR-129 or miR-383 did not reduce NET or glucocorticoid protein levels. These results indicate that both miR-181a and miR-29b are potentially relevant miRNAs to NET and GRs.

### **Overexpression of miRNAs 181a and 29b inhibited [<sup>3</sup>H] NE uptake in PC12 cells**

The uptake of [<sup>3</sup>H] NE assay was performed to evaluate whether over-expression of miR-181a or miR-29b was associated with a change of the ability of cells to transport NE. PC12 cells were transfected with mimics of miR-181a or miR-29b for 48 hours. The uptake assays showed that both miRNAs significantly reduced the uptake of [<sup>3</sup>H] NE ( $F_{2, 14}=6.27$ ,  $p<0.01$ ; Figure 3).

### **MiR-181a and miR-29b decreased the gene expression by direct binding to 3'UTR of the NET and GR**

To prove that downregulation of NET and GR protein levels is indeed caused by a direct interaction of miR181a and miR-29b with the 3'UTR of NET and GR mRNA, a putative target sites on 3'UTR of NET was identified by the computational analysis. As illustrated from Fig. 4, the NET 3'UTR fragments containing putative miRNA binding sites for miR-181a were cloned to multiple cloning site at downstream of firefly luciferase gene that is under control of the cytomegalovirus (CMV) promoter in pmirGLO reporter plasmid. Cloning orientation was verified by diagnostic cuts followed by sequencing. PC12 cells were

then co-transfected with 100  $\mu$ M of either miR-181a or miR-29b, and 100 $\mu$ g/L pmirGLO-NET3'UTR plasmid, or empty pmirGLO (as the control). Forty-eight hours after transfection, luciferase reporter assays from cell preparations were performed and results revealed that both miR-181a and miR-29b significantly reduced luciferase activity ( $F_{2,14}=3.98$ ,  $p<0.05$ , Fig. 5A), indicating that *NET* is direct target of miR-181a and miR-29b. The same procedure was also used for GR gene and a similar result validated GR similarly is the direct target of miR-181a and miR-29b (Fig. 5B). These results verify that miR181a and miR-29b downregulated the gene expression of the NET and GR by binding to the 3'UTR of NET or GR mRNAs.

### Corticosterone represses the expression levels of endogenous miR-29b

The investigation of the effect of corticosterone on expression of endogenous miR-181a and miR-29b was performed. PC12 cells were exposed to 100 nM corticosterone for 7 days, after which q-PCR from cell preparations showed that corticosterone treatment significantly reduced miR-29b in PC12 cells (Fig. 6A,  $p<0.01$ ). In a separate experiment, PC12 cells were exposed to 100 nM corticosterone or 100 nM corticosterone plus 5  $\mu$ M mifepristone (RU486), a GR antagonist (Cadepond *et al.* 1997) for 7 days. The measurement results showed that mifepristone neither affects the expression of miR-29b, nor influences corticosterone-induced repression of miR-29b expression (Fig. 6), indicating that alteration of endogenous miR-29b caused by corticosterone is GR-independent. However, although it was reported that miR-181a is expressed in PC 12 cells (Hamada *et al.* 2012, Liu *et al.* 2013), our preliminary study using real-time PCR could not prove it. Therefore, PC12 cells were transfected with miR181a mimic and then exposed to 100 nM corticosterone for 7 days. Real time PCR measurements showed that in this transfected PC12 cells corticosterone treatment did not cause any significant alteration in the extraneously introduced miR-181a level (data not shown).

### Discussion

In the present study we used *in silico* analysis to predict several miRNAs that may commonly bind mRNAs of NET and GR, leading to reduction of their activities. Our experimental results showed that overexpression of either miR-181a or miR-29b significantly reduced protein levels of NET and GR in PC12 cells, and this downregulation of NET is accompanied by a functional decrease in NET activity as indicated by a reduction of [<sup>3</sup>H]NE uptake. Further experiments verified that both miR-181a and miR-29b are able to bind the predicted seed region in the 3'UTR of NET and GR, and exposure of cells to corticosterone markedly repressed the expression of endogenous miR-29b, which was not blocked by application of GR antagonists. These findings reveal that the expression of NET and GR can be commonly regulated by miR-181a and miR-29b, and that endogenous miR-29b is also downregulated by stress hormones. This observation indicates that these two miRNAs may serve as the intermediaries in stress-induced upregulation of the noradrenergic phenotypes, and may play an important role in the development and treatments of depression (Goddard *et al.* 2010).



As a recently discovered class of noncoding RNA, miRNAs have been implicated in diverse biological processes and may act as modulators of several brain processes. Among these miRNAs, miR-181 family is expressed in mature neurons across most vertebrates (Miska *et al.* 2004, Smith *et al.* 2010), especially with a high concentration in the brain (Chen *et al.* 2004, Saba *et al.* 2012b), indicating its potential regulatory roles in the central nervous system. Recent studies demonstrated that as a multifunctional miRNA, miR-181a has been found to target the Bcl-2 family (Ouyang *et al.* 2012a), a protein that can protect from cerebral ischemia (Lawrence *et al.* 1996); the heat shock protein GRP78 (Ouyang *et al.* 2012b), transcription factor CREB1 (Liu *et al.* 2013), and glutamate receptor 2 subunit (GluA2) (Saba *et al.* 2012a). As such, it is potentially involved in neuronal protection against cerebral ischemia (Ouyang *et al.* 2012b, Ouyang *et al.* 2012a), drug addiction-related synaptic changes in the hippocampus (Saba *et al.* 2012b), the memory performance associated with epilepsy (Liu *et al.* 2015), and in Parkinson's disease (Ding *et al.* 2016). On the other hand, miR-29 family is also involved in translational repression of a wide range of target genes. It has been reported that members of the miR-29 family behave as epi-miRNAs by down-regulating important drivers of the epigenetic machinery, and many studies found that one function of miR-29 family is related to the cancer epigenetic (Amodio *et al.* 2015). However, increased expression of miR-29b was found in the brain of aged mice (Fenn *et al.* 2013), and its expression was significantly down-regulated in the blood serum of Alzheimer's disease patients (Geekiyanaige *et al.* 2012) and after stroke (Khanna *et al.* 2013). miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis (Kole *et al.* 2011) and regulates ethanol-induced neuronal apoptosis in the developing cerebellum (Qi *et al.* 2014). Our present study indicates that both miR-181a and miR-29b repress protein levels of NET and GR by directly targeting 3'UTR of these two genes. Furthermore, exposure of cells to corticosterone reduced the endogenous levels of miR-29b. Based on our knowledge, this is the first study reporting the involvement of both miRNAs in the regulation of NET and GR, which adds a new evidence for the notion that miRNAs are important regulators for neuronal homeostasis.

Glucocorticoids are the prominent mediators of cellular stress on neuronal function and behavior, and are also known to structurally alter brain cytoarchitecture (Hunsberger *et al.* 2009). It has been shown that treatment of cells with glucocorticoids activates gene transcription, a primary mechanism for increased expression of the target genes (Eberwine *et al.*, 1984; 1987; Karin, 1998). As a nuclear receptor, the GR not only mediates stressful effects of glucocorticoids, but also can act as a ligand-dependent transcription factor to influence gene expression (Aoyagi & Archer 2011). Our previous study showed that glucocorticoids bind to GR and activate another transcription factor c/EBP- $\beta$  to transactivate the transcription of NET (Zha *et al.* 2011). Based on the biological characteristics of miRNAs, their regulatory effects may also be involved in this regulation. First, miRNAs influence complex gene networks and cellular processes more quickly, relatively specifically, and effectively (Kosik 2006, Soifer *et al.* 2007, Hansen & Obrietan 2013, Wang *et al.* 2014). Second, miRNAs can modulate stress response and associated neuronal function (Leung & Sharp 2010). The psychological stress significantly alters the expression of miRNAs in the brain (Zucchi *et al.* 2013). For example, the expression of numerous miRNAs in the hippocampus, amygdala (Meerson *et al.* 2010), thymus (Belkaya *et al.* 2011)

and frontal cortex (Rinaldi *et al.* 2010, Babenko *et al.* 2012) of experimental animals was differentially altered after stress. Among these altered miRNA levels caused by stress, miR-181 family is one of most stress-responsive miRNAs. In mice injected with lipopolysaccharide, an infection stress inducer, miR-181d exhibited a 15-fold decrease while miR-181a and miR-181b are reduced 2- and 6-fold, respectively in the thymic tissue (Belkaya *et al.* 2011). Downregulated miR-181a increases the survival of astrocytes from ischemia-like injury following glucose deprivation (Ouyang *et al.* 2012a). As mentioned above, in our previous studies chronic stress and glucocorticoid treatments significantly increased the expression of NET (Sun *et al.* 2010, Zha *et al.* 2011, Chen *et al.* 2012), and GR (Zha *et al.* 2011), which is consistent with the reports from other laboratories (Froger *et al.* 2004, Li *et al.* 2011, Guidotti *et al.* 2013). In the present study, neither did we identify the expression of miR-181a in PC12 cells, nor did we find any significant expressional change of miR-181a after treatment with corticosterone in PC12 cells transfected with miR-181a mimics. Currently we do not have satisfactory explanation for failing to detect endogenous miR-181a in PC12 cells, with the only possible reason that its expression is lower than the detectable level in the present technical conditions of our laboratory. However, exposure of cells to corticosterone significantly reduced expression of endogenous miR-29b, indicating that this miRNA is negatively regulated by glucocorticoids. Together with the observation that miR-29b significantly represses protein levels of NET (Fig. 1), it suggests that this miRNA may be involved in the upregulation of NET caused by treatment with glucocorticoids. As an increased expression of NET may allow a homeostatic stress response to evolve into a pathological stress (Goddard *et al.* 2010), our current observation may be of pivotal importance for study regarding possible correlation between stress and development of depression. However, more studies are required to seek the direct evidence for that there is a relationship between the effect of corticosterone on miR-29b and the up-regulation of NET expression by corticosterone.

To further explore whether GR is involved in the negative regulation of corticosterone on endogenous miR-29b, GR antagonist mifepristone was administered in the experiment. The results show that blockage of GR cannot reverse the repressive effect of corticosterone on miR-29b (Fig. 6B), indicating the effect of corticosterone on miR-29b in PC12 cells is GR-independent, which is in conflict with the only other report that mifepristone blocked glucocorticoid-induced increase of miR-29c in rat aortic smooth muscle cells (Chuang *et al.* 2015). While use of different cell lines may be an explanation for this discrepancy, different action mechanisms of glucocorticoids can be accounted for. It is known that glucocorticoids act through both genomic and non-genomic mechanisms. Generally, the genomic effect of glucocorticoids can be achieved by binding to GRs, and activation of GRs in turn regulates gene expression (Rousseau *et al.* 1972). However, glucocorticoids can exert many important effects on the nervous systems through non-genomic pathways, by which the effect of glucocorticoids might be mediated through nonspecific interactions with intracellular machinery, rather than through GR signaling (Cato *et al.* 2002, Song & Buttgerit 2006), although the underlying mechanisms remain to be elucidated (Makara & Haller 2001). The non-genomic effects of glucocorticoids can be found in the inhibition of ion channels (Qiu *et al.* 1998, Lovell *et al.* 2004), CRF-induced ACTH secretion (Hinz & Hirschelmann 2000), activation of MAPKs pathways (Xiao *et al.* 2005), reduction of gonadotropin-releasing

hormone (GnRH) pulse (Wagenmaker *et al.* 2009), catecholamine release in chromaffin cells (Park *et al.* 2008), and upregulation of SP-B mRNA (Tillis *et al.* 2011). Given the study about miRNAs is still an emerging field, the non-genomic mechanism underlying the regulation of corticosterone on miR-29b in the present study can be a possibility.

It is noteworthy that expression and function of NET is closely related to the action mechanisms for some types of antidepressants with characteristics of tricyclic structures or specific inhibition of norepinephrine reuptake. Also, the therapeutic action of some antidepressants has been thought to be mediated, at least in part, by changing GR functional status, and blocking the GR represents a potential treatment strategy for depression (Anacker *et al.* 2011). Likewise, miRNAs may have similar activity as accumulating evidence reveals that they may be critical for the pathophysiology of depressive disorders by taking part in monoamine signaling (Dwivedi 2011b, Millan 2011) and psychotropic drug effectiveness (Baudry *et al.* 2010, Hansen & Obrietan 2013). It has been reported that antidepressant treatment significantly increased blood levels of 28 miRNAs (Bocchio-Chiavetto *et al.* 2013). In animal study, treatment with antidepressants ketamine and electroconvulsive shock therapy also increased miRNA expression in the hippocampus of stress animals (O'Connor *et al.* 2013). Interestingly, miR-29b is one of these miRNAs increased by antidepressant treatments. The present study identifies two miRNAs that commonly repress NET and GR expression, which suggests that miRNAs may be a possible mechanism regulating the protein levels involved in antidepressant actions.

In conclusion, our data suggest that stress and stress-released glucocorticoids may downregulate expression levels of miR-29b in the brain, which erase or reduce their normal repressive effects on NET and GR, and in turn increase the expression of NET and GR, as observed in our previous studies. Further elucidation of their roles *in vivo* may contribute to the investigation of mechanisms related to stress and antidepressant action. Furthermore, the data from present study provide *in vitro* evidence for an interaction between miRNA and NET. Downregulation of NET expression may be more effective than pharmaceutical treatments because of miRNA's high potential as mentioned above. The novel miRNAs, mir181a and mir29b, can help us to understand mechanism of interaction between miRNAs and monoamine transporters and to look for possibilities of using them for treatment of psychiatric and neurological disorders. However, the understanding of miR-181a and miR-29b is still fragmented. More investigations are warranted.

## Acknowledgements

This work is supported by NIH grant MH080323.

## Abbreviation

<b>GR</b>	glucocorticoid receptors
<b>HPA</b>	hypothalamic-pituitary-adrenocortical
<b>KRH</b>	Krebs-Ringer-HEPES buffer
<b>LC</b>	locus coeruleus

<b>miRNAs</b>	microRNAs
<b>NE</b>	norepinephrine
<b>NET</b>	norepinephrine transporter
<b>ODs</b>	optical densities
<b>PBS</b>	phosphate-buffer saline
<b>q-PCR</b>	quantitative real-time polymerase chain reaction

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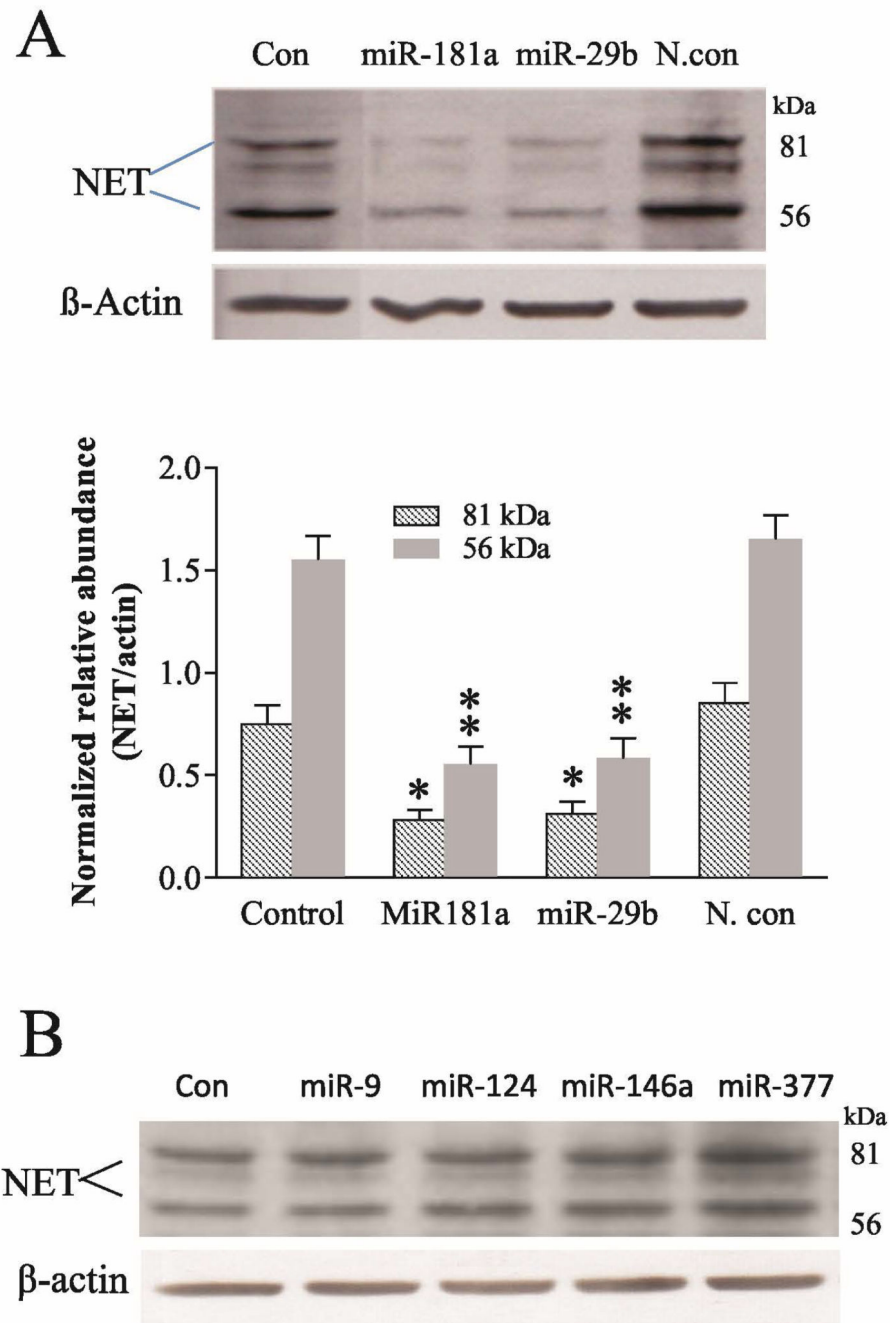
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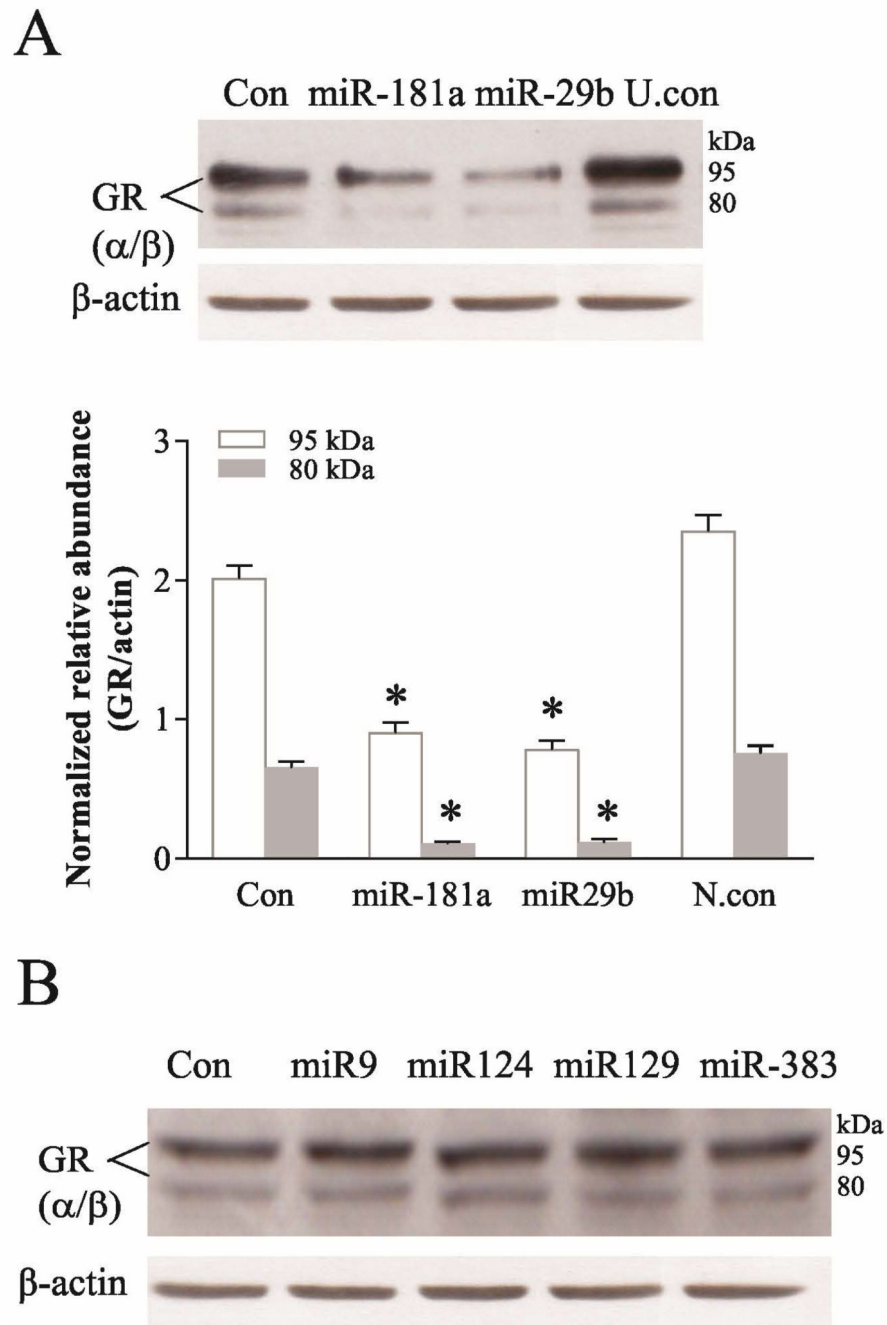
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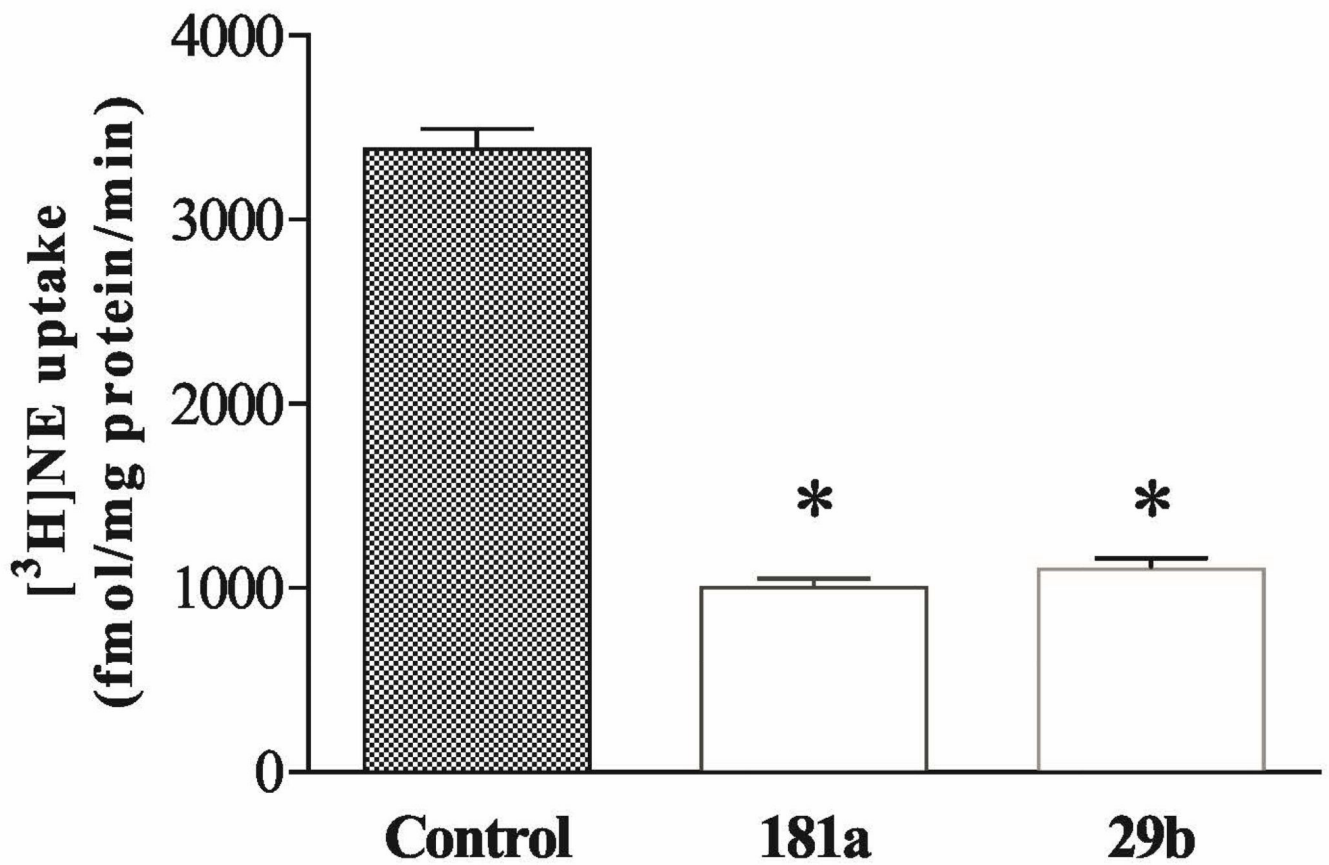




**Fig. 1.** Overexpression of miR-29b and miR-181a reduced NET expression in PC12 cells. A: Western blot measurements show NET protein levels after transfection of cells with mimics of miR-181a and miR-29b. Each bar in the quantitative analysis (the lower panel of A) represents data obtained from 5 separate experiments. B: Similar measurements show NET protein levels after transfection of cells with mimics of miR-9, miR-124, miR-146a and miR-377. Con: mimic control; N.con: negative control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , as compared to the corresponding mimic control.



**Fig. 2.** Overexpression of miR-29b and miR-181a reduced GR expression in PC12 cells. A: Western blot measurements show GR protein levels after transfection of cells with mimics of miR-181a and miR-29b. Each bar in the quantitative analysis (the lower panel of A) represents data obtained from 5 separate experiments. B: Similar measurements show GR protein levels after transfection of cells with mimics of miR-9, miR-124, miR-129 and miR-383. Con: control; N.con: negative control. \*  $p < 0.05$ , as compared to the control.



**Fig. 3.** Overexpression of miR-181a and miR-29b reduced [<sup>3</sup>H]NE uptake in PC12 cells. Cells were exposed to 100 nM corticosterone for 7 days. [<sup>3</sup>H]NE uptake assays were performed and each bar represents data obtained from 5 separate experiments. \*  $p < 0.01$ , compared to the control.

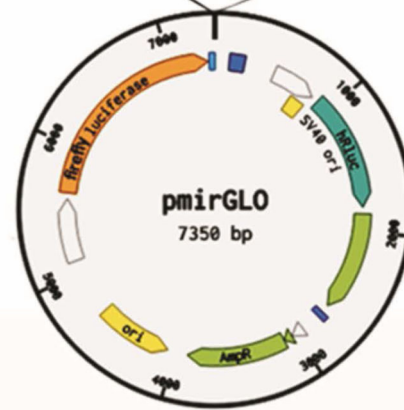
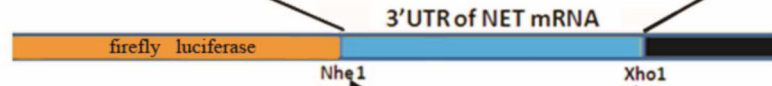
predicted target

Position 700-706 of SLC6A2 3' UTR	5' ...AUGAUGUUAGGGGGGA <b>GAAUGUAA</b> ...
<a href="#">rno-miR-181a</a>	3' UGAGUGGCUGUCGCAA- <b>CUUACAA</b>

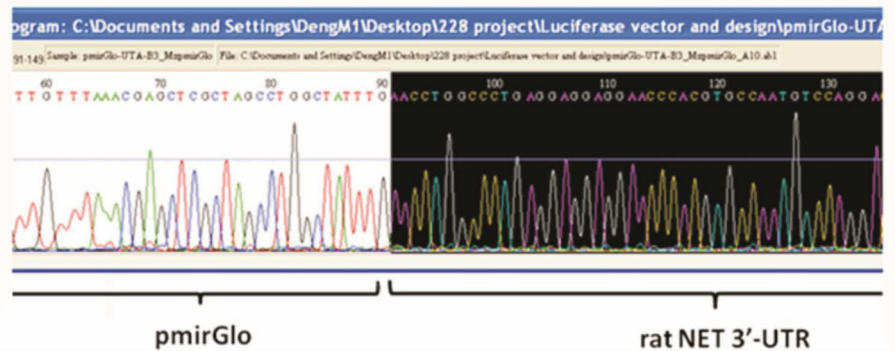
Rat SLC6A2 3' UTR



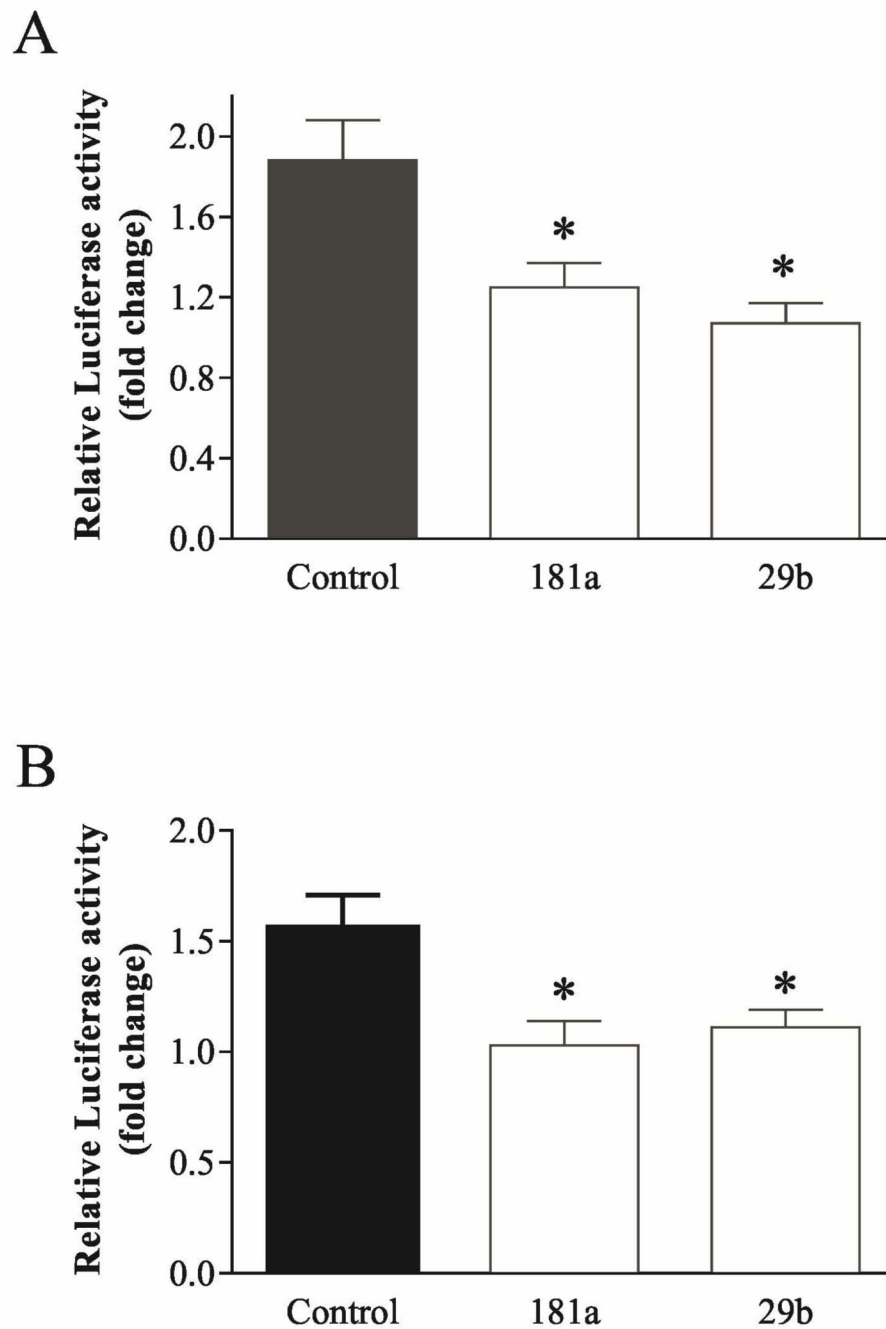
3'-UTR insertion



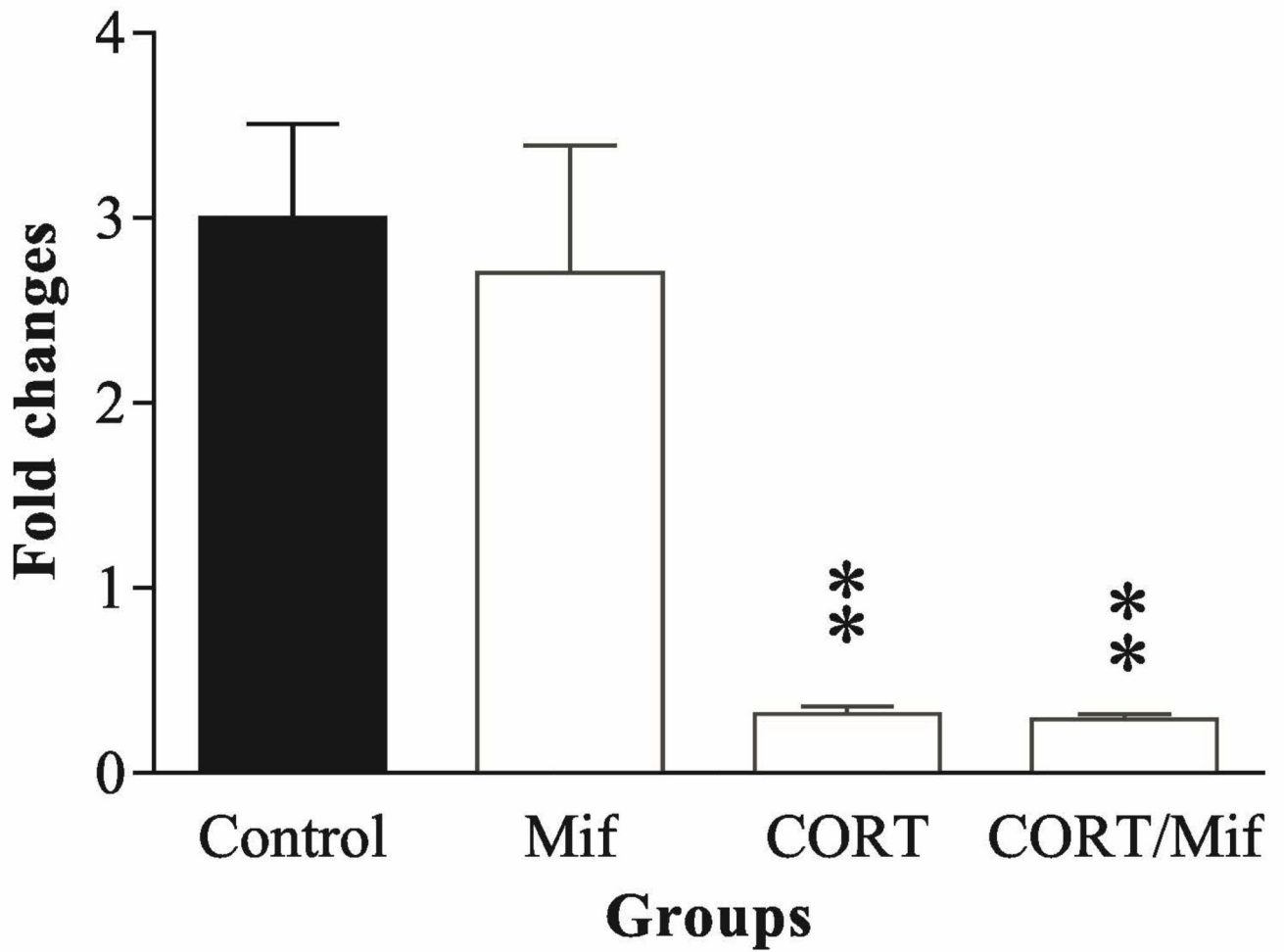
sequencing report



**Fig. 4.** Schematic shows the process to the analysis of the miR-181a and miR-29b binding site in 3'UTR region of NET mRNAs and clone 3'UTR of NET into pmirGlo vector to measure the luciferase activity.



**Fig. 5.** Confirmation of direct binding of miR-181a and miR-29b to 3'UTR region of NET (A) and GR (B) mRNAs by luciferase assay. The bars represent relative activity of luciferase normalized by renilla from 5 separate experiments. \*  $p < 0.05$ , compared to the control.



**Fig. 6.** Effects of exposing PC12 cells to 100 nM corticosterone for 7 days (A), or corticosterone plus 5 $\mu$ M mifepristone for 7 days (B) on levels of miR-29b. The bars represent fold changes from 4 separate experiments. \*\*  $p < 0.01$ , compared to the control. CORT: corticosterone, Mif: mifepristone.

**Table 1**

miRNAs are predicted to target mRNA transcript coding for NET.

miRNA	Spices	Position on SLC6A2-3'UTR	Sequential pairing	Conservation (%)
miR-29b	Rno	878-884	AGCACCAUU	100
miR-181a	Rno	712-718	ACAUUCA	100
miR-9	Rno	121-128	ACCAAAGA	100
miR-124	Rno	729-735	AAGGCAC	100
miR-146a	Rno	725-731	CCCUAGGGA	100
miR-377	Rno	1095-1101	GAGGUUG	100

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**Table 2**

miRNAs are predicted to target mRNA transcript coding for the glucocorticoid receptor.

miRNA	Species	Position on Nr3c1-3'UTR	Sequential pairing	Conservation (%)
miR-29b	Rno	1450-1457	AGCACCAUU	100
miR-181a	Rno	2177-2183	ACAUUCA	100
miR-9	Rno	1304-1310	UCGACAU	100
miR-124	Rno	20-26	AAGGCAC	100
miR-129	Rno	1532-1539	AGCCCUU	100
miR-384	Rno	436-442	GUAAAC	100

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