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Altered Expression of Phox2 Transcription Factors in the Locus Coeruleus in Major Depressive Disorder Mimicked by Chronic Stress and Corticosterone Treatment in Vivo and in Vitro

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Altered expression of Phox2 transcription factors in the locus coeruleus in major depressive disorder mimicked by chronic stress and corticosterone treatment *in vivo* and *in vitro*.

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Abstract

Phox2a and Phox2b are two homeodomain transcription factors playing a pivotal role in the development of noradrenergic neurons during the embryonic period. However, their expression and function in adulthood remain to be elucidated. Using human postmortem brain tissues, rat stress models and cultured cells, this study aimed to examine the alteration of Phox2a and Phox2b expression. The results show that Phox2a and Phox2b are normally expressed in the human locus coeruleus (LC) in adulthood. Furthermore, the levels of Phox2a protein and mRNA and protein levels of Phox2b were significantly elevated in the LC of brain donors that suffered from the major depressive disorder, as compared to age-matched and psychiatrically normal control donors. Fischer 344 rats subjected to chronic social defeat showed higher mRNA and protein levels of Phox2a and Phox2b in the LC, as compared to non-stressed control rats. In rats chronically administered oral corticosterone, mRNA and protein levels of Phox2b, but not Phox2a, in the LC were significantly increased. In addition, the corticosterone-induced increase of Phox2b protein was reversed by simultaneous treatment with either mifepristone or spironolactone. Exposing SH-SY5Y cells to corticosterone significantly increased expression of Phox2a and Phox2b, which was blocked by corticosteroid receptor antagonists. Taken together, these experiments reveal that Phox2 genes are expressed throughout the lifetime in the LC of humans and Fischer 344 rats. Alterations in their expression may play a role in major depressive disorder and possibly other stress-related disorders through their modulatory effects on the noradrenergic phenotype.

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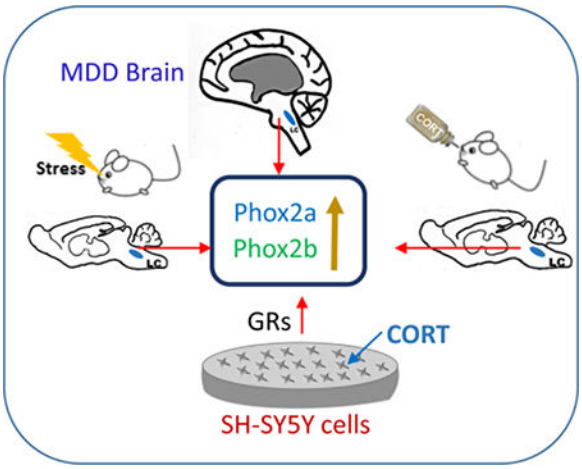
Graphical Abstract



Keywords

Phox2 genes; major depressive disorder; stress; corticosterone; postmortem; locus coeruleus

Introduction

Phox2a and Phox2b are two closely related homeodomain transcription factors with relatively similar expression patterns, and play a pivotal role in the specification and differentiation of noradrenergic neurons during the embryonic period (Morin *et al.* 1997, Hirsch *et al.* 1998, Pattyn *et al.* 2000). A large body of experiments demonstrated that the silencing of the *Phox2a* gene leads to the agenesis of the noradrenergic locus coeruleus (LC) in mice (Morin et al. 1997). Inactivation of *Phox2b* disturbs noradrenergic differentiation in both central and peripheral nervous systems (Pattyn *et al.* 1999, Pattyn et al. 2000). These important roles of the *Phox2a* and *Phox2b* on noradrenergic neurons are further revealed by their involvement in the transcriptional control of noradrenergic phenotypes such as expression of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). In fact, *Phox2a*$^{-/-}$ or *Phox2b*$^{-/-}$ embryos fail to express DBH (Morin et al. 1997, Pattyn et al. 1999), similar to that observed in *DBH* knock-out animals (Thomas *et al.* 1995, Pattyn et al. 2000). Forced expression of either *Phox2a* or *Phox2b* can induce TH *in vitro* (Lo *et al.* 1999, Stanke *et al.* 1999) and *in vivo* (Guo *et al.* 1999, VogelHopker & Rohrer 2002). These data establish Phox2 genes as determinants for development of the noradrenergic neuronal phenotype during embryogenesis.

Several studies demonstrate that *Phox2a* and *Phox2b* expression continues beyond embryonic development (Tiveron *et al.* 1996, Kang *et al.* 2007, Card *et al.* 2010). Functional investigations reveal that *Phox2a* and *Phox2b* are essential regulators of noradrenergic marker gene expressions past early development (Howard 2005). Their expression continues to be required for the control of adaptive homeostatic functions of mature neurons that contribute to brainstem networks (Pattyn et al. 2000, Tiveron *et al.* 2003). Our previous *in vivo* study showed that a significant reduction in mRNAs of *DBH* in the LC and adrenal

glands of aging rats was paralleled with a decline in mRNA levels of *Phox2a* and *Phox2b* (Zhu *et al.* 2005), supporting the importance of Phox2 genes to the expression of the noradrenergic phenotype well beyond birth. Furthermore, *in vitro* investigations demonstrate a direct transcriptional activation of TH and DBH by Phox2 genes (Kim *et al.* 1998, Yang *et al.* 1998b, Yang *et al.* 1998a, Seo *et al.* 2002, Fan *et al.* 2009). Together, these investigations strongly implicate a regulatory effect of *Phox2a* and *Phox2b* on the expression of noradrenergic phenotypes into adulthood.

The noradrenergic system plays a critical role in attention, learning and memory, emotion, sleep/wakefulness and central responses to stress (Berridge & Waterhouse 2003, Sara 2009). A disruption of this system is believed to have a close relationship with major depressive disorder (MDD) (Ressler & Nemeroff 1999, Chandley & Ordway 2012). Pathologically, MDD can exhibit a pronounced and sustained central hyper-noradrenergic function as indicated by increased TH protein in the LC of postmortem brains from donors who died by suicide (Ordway *et al.* 1994, Gos *et al.* 2008) and/or donors that suffered from MDD at the time of death (Zhu *et al.* 1999), as compared to matched control donors. Consistent with these findings, elevated norepinephrine concentrations in the cerebrospinal fluid paralleled by an increase of plasma cortisol has been observed in depressed patients (Wong *et al.* 2000, Gold *et al.* 2005). Chronic stress is a major factor contributing to elevated risk of developing MDD (Mundt *et al.* 2000, Mann & Currier 2010). Stress greatly activates the LC (Robbins & Everitt 1995), resulting in elevated norepinephrine release (Pavcovich *et al.* 1990). Animal studies have shown that stress significantly increases *TH* expression and TH protein in the LC and other noradrenergic nuclei (Nankova *et al.* 1996, Wang *et al.* 1998, Makino *et al.* 2002). Given the critical role of Phox2 genes on the noradrenergic phenotype, it is reasonable to expect that *Phox2a* and *Phox2b* may regulate noradrenergic adaptations in response to stress and in MDD. There is one report of chronic stress-induced alterations of *Phox2b* expression in the mouse adrenal medulla (Santana *et al.* 2015), but how the Phox2 genes in the CNS are regulated by stress or in stress-related disorders is unknown.

Based on the important role of Phox2 genes on the expressional and functional regulation of the noradrenergic phenotype, we hypothesize that the expression of phox2a and Phox2b in the central noradrenergic system is possibly altered under depressive and stress conditions. Phox2 gene expression changes may mechanistically underlie increases in markers of the noradrenergic phenotypes in the LC of depressive patients and under stressful condition (Melia *et al.* 1992, Nankova et al. 1996, Zhu et al. 1999, Fan *et al.* 2014). Therefore, the present study was designed to investigate whether the expression of *Phox2a* and *Phox2b* is altered in MDD in human, or by exposure to chronic stress in rodents. mRNA and protein levels of Phox2a and Phox2b were assessed in the LC of postmortem brains from MDD donors, and in brains of rats subjected to chronic social defeat (CSD) or treated with corticosterone (CORT). Furthermore, an *in vitro* study was performed to examine the expression of these genes after exposing cells to CORT. The results from the present experiments indicate that Phox2 genes may play an important role in MDD and in adaptation to stress through modulation of the noradrenergic phenotype.

Experimental procedures

Human tissue collection and section:

The methods for obtaining human brains tissues are exactly as described in the previous report (Zhu et al. 1999). Briefly, human brain tissues were collected from the Cuyahoga County Coroner's Office, Cleveland, Ohio, in accordance with an approved Institutional Review Board protocol for human studies. Information on the lifetime and current (within the last month) psychiatric status of all brain donors was obtained in structured clinical interviews with the next-of-kin by a trained interviewer for DSM-IV Axis I Disorders modified for third-person reporting (First et al. 1996). Brain tissues were collected from 12 donors diagnosed as having MDD at the time of death and from 12 matched control donors that had no Axis I diagnosis at the time of death or historically. From over 50 donors with MDD available from the brain collection, donors with MDD chosen for study were those that had the least number of potentially confounding factors (psychotherapeutic drugs in toxicology, low RNA quality, low tissue pH, comorbidities) and that could be matched for age and postmortem interval to available control donors that also had the least number of potentially confounding factors. The age of subjects ranged from 18–77 years with an average of 45 ± 5 years for control group (all males) and 48 ± 5 years for MDD group (all males). A summary of subject information as a result of psychiatry autopsy including a toxicology screen from all of the subjects is outlined in Table 1. All demographic data (e.g. ages) is not provided in the table to protect donor identities. Psychiatrically normal control and MDD donors were matched as closely as possible for age, postmortem interval (PMI), brain tissue pH and smoking history.

Human brains were frozen (unfixed) and blocking was the same as previously reported (Zhu et al. 1999). The floor of the fourth ventricle and the pons were its dorsal and ventral surfaces, respectively. The rostral surface was formed by a transverse cut immediately caudal to the inferior colliculus (at the frenulum). Tissue lateral to the superior cerebellar peduncles was trimmed away. Particular care was taken in the freezing process to maintain gross morphology. For example, the block of pontine tissue was dissected to form a flat surface on the ventral pontine surface of the LC tissue block. This surface was placed on a hard piece of cardboard, which was then lowered for 10 sec into 2-methylbutane cooled on dry ice to −50 °C for quick freezing. Tissue blocks were then placed on powdered dry ice for 10 min and then stored in an ultracold freezer (−80°C). When sectioning, tissue blocks containing the LC were paired (MDD donor and age-matched control donor) and the caudal surfaces of each pair were co-mounted to the specimen chuck of a cryostat microtome (Leica, Cryocut 1800, Deerfield, IL). In this way, a single MDD-control pair was sectioned simultaneously and paired sections were mounted on the same microscope slide to be processed concurrently throughout the experiment. This pairing procedure reduced the influence of factors that could artificially contribute to differences between MDD and normal control donors. Frozen tissue blocks containing the LC were cut transversely at the same anatomical level in all donors, at approximately the middle along the rostro-caudal axis the LC (approximately 5 mm caudal from the frenulum). Tissue sections (20 μm) were cut at −16°C and thaw-mounted onto gelatin-coated microscope slides, and stored at −80°C until assay.

Animals:

Male Fischer 344 rats weighing 200–250g at the beginning of the experiment, Long-Evans retired male breeders, and ovariectomized female rats were purchased from Harlan Laboratories Inc. (Indianapolis, IN, USA). All animal procedures were approved by the Animal Care and Use Committee of East Tennessee State University (approval reference number: P130701), and complied with the NIH Guide for the Care and Use of Laboratory Animals. Rats were maintained on a 12-h light/dark cycle (lights on at 07:00 h) with ad-libitum access to food and tap water except as specifically described below. They were housed in a group cage (up to 3 rats per cage except for breeders and ovariectomized female rats). After an acclimation period of 5 days, rats were randomly assigned to experimental groups, in which animals were identified and recorded using a unique code during experiments of CSD and CORT ingestion, biochemical measurements and analysis. ARRIVE guidelines were taken into consideration in experimental reporting. This study was not pre-registered.

Chronic social defeat (CSD) paradigm:

This protocol has been reported previously (Chen *et al.* 2012). Briefly, each pair of Long-Evans rats (larger retired male breeders and sterile female rats, residents) was housed together in individual cages for 7 days. On the 8^{th} day, the female was removed and an adult male Fischer 344 rat (intruder) was placed into the cage for 2 min. After being attacked and defeated, as shown by a supine and submissive posture, the “intruder” was rescued into a small protective cage within the resident’s cage, which precluded further physical contact, but allowing visual, auditory, and olfactory contact with the resident. The “intruder” was left in the cage of the “resident” for 1.5 h in the small enclosure. Some male Fischer 344 rats (controls) were transferred to the resident home cage when the residents had been removed. Therefore, these control rats were never physically attacked and defeated by the residents. The resident-intruder exposure was repeated 4 times in the first and fourth weeks, and 2 times in the second and third weeks. After the last session of CSD paradigm, rats were immediately sacrificed by rapid decapitation without anesthesia for biochemical measurements.

Oral administration with CORT and drug treatment:

This procedure is essentially as described previously (Fan et al. 2014). Briefly, male Fischer 344 rats were administered an oral solution containing CORT (Sigma, St. Louis, MO; 100 μg/ml solution in ad lib drinking bottles) at 9:00 am of each day for 21 days, which was freshly prepared daily. This dose of CORT, which is approximately 30 mg/kg/day (Gourley *et al.* 2008), results in an increased plasma CORT level approaching that observed during stress (Karatsoreos *et al.* 2010, Donner *et al.* 2012). Control rats were given the vehicle alone (a 2.4% ethanol solution used for preparation of CORT solution), which neither activates the hypothalamic-pituitary-adrenal axis, nor causes other biochemical alterations including organ weight (Magarinos *et al.* 1998, Nacher *et al.* 2004a, Nacher *et al.* 2004b, Gourley & Taylor 2009). A separate set of rats were injected with glucocorticoid receptor antagonist mifepristone (10 mg/kg, daily, s.c.; Sigma-Aldrich, St. Louis, MO, USA) or mineralocorticoid receptor antagonist spironolactone (15 mg/kg, daily, s.c.; Sigma-Aldrich,

St. Louis, MO, USA); either alone or in combination at a similar time (around 9:00 am). In addition, a separate set of rats was injected daily with either mifepristone or spironolactone without CORT ingestion. The selection of doses of these antagonists was based on previous reports (Ratka *et al.* 1989, Ni *et al.* 1995, Haller *et al.* 1998, Macunluoglu *et al.* 2008) and our preliminary pilot data (Fan et al. 2014). Rats in the untreated control and CORT alone groups were injected with vehicle. After oral administration of CORT and related compounds for 21 days, animals were sacrificed on the 22nd day for biochemical measurements.

In situ hybridization to measure Phox2 mRNAs.

The *in situ* hybridization method has been described previously (Zhu *et al.* 2002). After rats were sacrificed, brains were removed and rapidly frozen in 2-methyl-butane on dry-ice, then stored at −80°C until sectioning. Sections (16 μm, not in pairs) were cut through the pontine LC region on a cryostat, and were mounted on SuperFrost Plus slides (Fisher Scientific; Pittsburg, PA), and stored at −80°C until assay. When hybridization was performed, slide-mounted tissue sections were fixed with 4% (w/v) paraformaldehyde followed by acetylation (with acetic anhydride) and washing (with an alcohol solution). [^{35}S]-labeled cRNA probes were transcribed *in vitro* from cDNAs of *Phox2a* and *Phox2b* of human (for postmortem) or rats in pGEM-3Zf vectors with T3 RNA polymerase. The probe sizes for rat *Phox2a* and *Phox2b* were 0.85 and 0.95 kb, respectively, and for human *Phox2a* and *Phox2b* were 1.4 and 1.6 kb, respectively. The sequences of all these probes are located in the N-terminal of the genes. Pre-hybridized sections were incubated with radiolabeled probes followed by extensive washing, and then apposed to Biomax autoradiographic film (Kodak; Rochester, NY). The film exposure time is dependent upon the activities of radioprobes ranging from 24 to 48 hours, which was determined by the signal in the radioactivity meter measured on the slides after post-hybridization. For higher-resolution studies, sections were also dipped in Kodak NTB2 emulsion. The specificity of cRNA probes was tested using three criteria. First, sense probes synthesized from each cDNA were used to perform *in situ* hybridization in parallel with antisense probes. There were no specific signals on these slides. Second, antisense probes were used on control slides from the cerebellum and cortex and no hybridization signals were detected. Third, antisense probes were hybridized to slides that were treated with RNase A (20 μg/mL) and no hybridization signal was detected.

The same quantification method was performed for both rat and human. For analysis of *in situ* hybridization results, 3 sections from each rat and bilateral LC regions from each section were quantitated. Optical densities of developed autoradiograms were quantified using an image analysis system (MCID M2; Imaging Research Inc., Ontario, Canada). Relative abundance of Phox2^{+} silver grains are represented in optical density units, estimated by calibrating the image analysis system with an optical density stepwedge (Eastman Kodak Co., Rochester, New York). The compact cell body region of the LC was drawn on the image and optical densities within this drawing were recorded. Optical densities of Phox2 gene expressions were measured in a range where there was a linear relationship between Phox2a and Phox2b mRNA amounts and optical density of the film, confirmed with a set of calibrated recombinant Phox2 standards spotted onto Immobilin-P membrane. The optical density of background, i.e., representing areas of the membrane that

were hybridized, but where no tissue was transferred, were very low (averaging 0.06 optical density unit).

Immunocytochemical staining:

The immunocytochemical staining procedures are the same as previously reported (Zhu *et al.* 2007). Briefly, human brain sections were pre-incubated in 5% bovine serum in phosphate buffer saline (PBS) supplemented with 0.2% Triton-X 100 for 1 h, followed by incubation in primary antibodies (1:200 dilution, monoclonal AB against Phox2a, RRID: AB_547013 and polyclonal AB against Phox2b, RRID:AB_10889846, both from Novus Biologicals, Littleton, CO, USA) overnight. On the following day, binding of Phox2a and Phox2b antibodies was detected with a biotinylated secondary antibody using the ABC kit (Vector Laboratories, Burlingame, CA) based on the manufacturer's instructions. 3, 3'Diaminobenzidine tetrahydrochloride (DAB) was used as the substrate. Staining for Phox2immunoreactivity (ir) was then visualized and semi-quantitatively analyzed using ImageJ software (Rasband, US National Institutes of Health, Bethesda, http://rsbweb.nih.gov/ij, 2010). Briefly, images were acquired in an Olympus BX41 microscope (Tokyo, Japan) equipped with an Olympus U-TVO digital camera connected to a computer with MagnaFire image software (Goleta, CA). Three digital microscopic images under higher power (40x objective lens) were randomly captured of the cell body region of the LC. The image size of the analysis field was ~75 μm^2. These grey-scale images were thresholded in ImageJ with a fixed level over the background, and this threshold was used for all images. The threshold values for Phox2-ir were obtained by manually sampling the signal intensity in each image, which was visually compared with the original grey-scale images to ensure that the tool effectively resolved all labeled cells. Thereafter, the intensity of Phox2-ir in the area was semi-quantitatively obtained using the measurement tool of ImageJ and the target (above threshold) area was expressed as a percentage of the sampled area. Two sections from each animal were examined. The average of the measurements (percent areas) obtained in 12 MDD donors from each group was a reflection of staining intensity for the LC.

Detection of protein levels:

Protein levels of Phox2a and Phox2b from rat LC and cultured cells were determined by western blotting as reported previously (Fan *et al.* 2011). Briefly, these samples were prepared by homogenization, centrifugation and measurement of protein concentrations. Then, equal amounts of samples (20 μg of protein per lane) were loaded on 10% SDS-polyacrylamide gels for electrophoresis. Electro-blotting was performed to transfer proteins in the gels to polyvinylidene diflouride membranes, which were incubated with primary antibodies against rat Phox2a (1:500 dilution; rabbit polyclonal; Abcam, Cambridge, MA, USA; RRID: AB_130121) or Phox2b (1:500 dilution; rabbit polyclonal; Abcam, Cambridge, MA, USA; RRID: AB_183741) overnight and further with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG, 1:3000; Amersham Biosciences, Little Chalfont, UK). Immunoreactive bands were visualized and detected by G:Box Imaging (Fyederick, MD, USA), or exposed on films and scanned by a Quantity One imaging devices (Bio-Rad, Hercules, CA). Band densities were then quantified by imaging software (Molecular Dynamics IQ solutions, Molecular Dynamics, Inc., Sunnyvale, CA). OD values of Phox2a and Phox2b signals were normalized with β-actin immunoreactivities, determined

on the same blot. Normalized values were then averaged for all gels from separate animals (N numbers) of replicated experiments, and used to calculate the relative changes on the corresponding gel, presented as means ± SEM as described in 2.10.

To validate the antibodies, before the experiment, the antibody specificity was tested by using blocking peptides and results showed there was no band at the known molecular weight for the target after addition of the blocking peptide. Also, a linear standard curve was created from optical densities (ODs) of bands with a dilution series of total proteins prepared from cells. OD values of Phox2a or Phox2b were compared with those of the standard curve to ensure that detection was in the linear range of measurement. Thereafter, quantitated values were normalized with β-actin immunoreactivities determined on the same blot to assess equal protein loading. The value normalization is the same as described in above section.

Cell culture and drug exposure:

The human neuroblastoma cell line SH-SY5Y (from ATCC, Cat#: CRL-2266, RRID: CVCL_0019) was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37°C in humidified air containing 5% CO2 as described previously (Wang 2013). FBS was replaced by dialyzed FBS (Gibco-Invitrogen, Carlsbad, CA), when cells were treated with CORT. Drug exposures were started at day 3, when each subculture had become confluent. CORT (Sigma, St. Louis, MO), dissolved in 40 μl DMSO and then further diluted with saline, was added to 6-well plates in fresh medium that was changed daily. The same amount of vehicle was added into the drug-free medium for cells in the control group. Based on our previous study (Sun *et al.* 2010) and a preliminary experiment of this study, cells were exposed to different concentration of CORT for 3 days. Then cells were harvested after washing twice with fresh, ice-cold PBS and immediately lysed to obtain total proteins. In addition, SH-SY5Y cells were exposed to CORT plus either mifepristone (5 μM) or spironolactone (5 μM, both from Sigma-Aldrich, St. Louis, MO, USA) for 3 days. The selection of these concentrations of receptor antagonists was based on the literature, in which 1–10 μM of mifepristone or spironolactone was reported to fully block corticosteroid-induced biological effects *in vitro* (Xiao *et al.* 2000, Son *et al.* 2001, Golde *et al.* 2003, Pickering *et al.* 2003).

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) analysis for mRNA of Phox2a and Phox2b.—The method is the similar as reported previously (Zha *et al.* 2011). Briefly, total RNA was extracted using RNAzol reagent (Molecular Research Center, Inc., Carlsbad, CA) from harvested cells after SH-SY5Y cells were exposed to 100 nM CORT for 3 days. Then cDNAs were converted using the superscript III First-Strand Synthesis Kit (Applied Biosystems/Life technologies, Forster City, CA) according to the manufacturer's protocol. Real-time PCR was conducted using the SYBR green Platinum Quantitative PCR supermix (Invitrogen, Carlsbad, CA). The primers for q-PCR were as follows: human *Phox2a*: forward 5'-CAT TTA CAC GCG TGA GGA GCT GGC-3' and reverse 5'- TCC TGT TTG CGG AAC TTG GCC C-3'. Human *Phox2b*: forward 5'-CAC CCT CAG GGA CCA CCA GA-3' and reverse 5'-TTC TCG TTG AGG

CCG CCG T-3'. Human β-*actin* (*ACTB*): forward 5'-TGT GCC CAT CTA CGA GGG GTA TGC-3' and reverse 5'-GGT ACA TGG TGG TGC CGC CAG ACA-3'. A standard curve was generated by analysis of the serial dilutions of β-*actin* oligo fragment solutions (10^2–10^7 copies/μl). For each sample, the copy number of *Phox2a*, *Phox2b* and *β-actin* was extrapolated from their respective standard curves. The value of Phox2a and Phox2b gene expression was normalized with the β-actin copy number and expressed in arbitrary units. Reproducibility of results was determined by triplicate measurements of each cDNA aliquot, each using separate sets of samples.

Statistics:

The sample size of rats used in CSD and CORT administration were estimated by power analysis. It was calculated that to achieve 95% power ($p<0.05$), a sample size of 7–8 animals per group was required. All experimental data are presented in the text and graphs as the mean ± SEM. Data were analyzed by a paired *t*-test (for human postmortem study), unpaired *t*-test (animal and cell culture studies), or one way analysis of variance (ANOVA, SigmaStat, Systat Software Inc., Richmond, VA) when multiple treatment groups were compared. A *post-hoc* Student-Newman-Keuls test was performed for planned comparisons of multiple groups. Pearson correlations related to human tissues were computed for potential relationships between age or other possible modifiers [brain pH, postmortem interval (PMI), RNA quality assessed by RNA integrity number (RIN)] and dependent variables (levels of Phox2a and Phox2b mRNAs and proteins).

Results

Expression of Phox2a and Phox2b in the LC of MDD.

In situ hybridization was performed to examine mRNA levels of *Phox2a* and *Phox2b* in the LC of brain donors that had MDD and matched control donors that were psychiatrically normal (n=12/group for both groups). Grain densities of Phox2b mRNAs in MDD donors were 28% greater (Fig. 1B, $t=3.16$, $p= 0.019$) than that of the control donors. Although *Phox2a* expression levels were modestly increased, this was not statistically significant (Fig. 1A). Using brain tissues cut from same MDD/control pairs used for *in situ* hybridization, Phox2a and Phox2b protein levels were measured immunocytochemically. In MDD donors, levels of Phox2a-ir and Phox2b-ir in the LC region were elevated by 16% (Fig. 2A, $t=2.37$, $p=0.035$) and 23% (Fig. 2B, $t=2.98$, $p=0.021$), respectively, as compared to control donors. Pearson correlation analyses showed that except for a modest correlation between levels of Phox2b-ir and age ($R=0.434$; $p=0.034$), there were no other significant correlations between brain pH, PMI, RIN, or age and levels of Phox2 mRNAs or proteins (Table 2). A One-way ANCOVA analysis was conducted to determine whether age impacted the group effect (MDD vs control) on Phox2b-ir. Age did not alter the interpretation of the results; the ANCOVA with age as the covariate chowed a significant difference between the MDD and control groups for Phox2b-ir ($F_{1,\ 23}=12.66$, $p=0.002$).

Expression of Phox2a and Phox2b in the LC of rats subjected to CSD.

Ex vivo in situ hybridization was performed to examine effects of CSD on the mRNA levels of *Phox2a* and *Phox2b* in the LC of rats. As show in Fig. 3A, exposing to CSD significantly

increased *Phox2a* expression by 76% (t=4.56, p<0.01), and *Phox2b* expression by 34% (t=3.07, p<0.05), compared to unstressed control rats. Western blotting was performed to determine levels of Phox2a-ir and Phox2b-ir in the LC from the CSD and control groups. Exposing to CSD significantly increased levels of Phox2a-ir by 34% (t=3.29, p<0.05) and Phox2b-ir by 21% (t=2.99, p<0.05), as compared to unstressed control rats (Fig. 3B).

Expression levels of Phox2a and Phox2b in the LC of rats treated with oral CORT.

To further evaluate stress-related changes in the expression of *Phox2a* and *Phox2b*, as well as their proteins in the LC, rats were orally administered CORT (~30 mg/kg/day) via drinking water for 3 weeks. Although CORT treatment resulted in a significant increase in *Phox2b* expression (by 66%, t=4.01, p<0.01), it failed to significantly change Phox2a mRNA levels in the LC, as compared to the control group (Fig. 4A). Western blotting of Phox proteins revealed results parallel to *in situ* hybridization. Treatment with CORT enhanced levels of Phox2b-ir in the LC by about 26% (t=3.02, p<0.05). However, the same treatment did not significantly influence Phox2a-ir levels in the LC (Fig. 4B).

To examine whether the altered Phox2b protein levels caused by administration of CORT were related to corticosteroid receptors, mifepristone or spironolactone, alone or in combination, were administered to rats together with oral administration of CORT for the same period. Western blotting analysis exhibited a significant influence of these treatments on Phox2b-ir levels ($F_{3,34}$ = 4.18, p<0.05, Fig. 5). Simultaneous treatment with either mifepristone or spironolactone prevented the CORT-induced increase in Phox2b-ir levels in the LC. However, there were no additional effects when both corticosteroid receptor antagonists were administered together (Fig. 5). In addition, treatment with either mifepristone or spironolactone alone (without oral administration of CORT) did not significantly affect Phox2b-ir levels (data not shown).

Phox2a and Phox2b in the SH-SY5Y cells exposed to CORT.

To verify whether CORT affects the expression of *Phox2a* and *Phox2b in vitro*, SHSY5Y cells were exposed to different concentrations of CORT for 3 days. The selection of this cell line is based on characteristics that closely mimic noradrenergic neurons with respect to display of the noradrenergic phenotype and to catecholamine metabolism (Balasooriya & Wimalasena 2007, Xicoy *et al.* 2017). Western blotting analysis showed that CORT significantly increased levels of Phox2a-ir and Phox2b-ir in a concentration-dependent manner ($F_{4,19}$ = 4.67, p<0.05 for Phox2a; $F_{4,19}$ = 4.32, p<0.05 for Phox2b, Fig. 6). Post-hoc tests showed exposure of cells to 100 and 500 nM of CORT significantly elevated Phox2a-ir by 35% and 46%, and Phox2b-ir by 34% and 52%, respectively (all p<0.05). Based on the observation of the concentration-response to CORT in western blotting, mRNA levels of *Phox2a* and *Phox2b* were examined after cells were exposed to 100 nM CORT for 3 days. Real time PCR analysis demonstrated that mRNA levels of both *Phox2a* (increased by 23%, p<0.05) and *Phox2b* (increased by 93%, p<0.001) were significantly enhanced by treatment with CORT (Fig. 7).

Further, SH-SY5Y cells were simultaneously exposed to 100 nM CORT alone, and 100 nM CORT plus either 5 μM mifepristone, 5 μM spironolactone, or both, as well as 5 μM

mifepristone and 5 μM spironolactone without CORT for 3 days. Western blotting analysis showed that these treatments significantly affected levels of Phox2-ir and Phox2b-ir ($F_{6,27}$ = 3.89, p<0.05 for Phox2a; $F_{6,27}$ = 4.05, p<0.05). Further analysis revealed that while neither mifepristone or spironolactone alone did significantly affected levels of both Phox2a-ir and Phox2b-ir, these corticosteroid receptor antagonists significantly blocked CORT-induced increases of Phox2 protein levels (both p<0.05, Fig. 8). There is no additional effect when cells were exposed to CORT in combination with both mifepristone and spironolactone.

Discussion

The most striking finding of this study is that there were elevated protein levels of Phox2a and Phox2b, as well as elevated mRNA levels of *Phox2b* in the LC of MDD donors , possibly contributing to the psychopathology of MDD. As the development of MDD is closely related to psychological stress exposure (Mundt et al. 2000, Mann & Currier 2010), the expression of these genes were also examined in the rats either exposed to CSD or treated with the stress hormone CORT. The results show that either CSD exposure or treatment with CORT similarly increased Phox2b mRNA and Phox2b protein levels in the LC, while CSD also increased Phox2a protein levels in the LC. Furthermore, exposure of SH-SY5Y cells to CORT upregulated the expression of Phox2a and Phox2b. Mifepristone and spironolactone reversed CORT-induced upregulation of Phox2b proteins *in vivo* and *in vitro*, indicating that corticosteroid receptors mediate CORT-induced changes in Phox2b proteins. Together, these experiments reveal the presence of Phox2a and Phox2b in the mature human brain and a potential role of these transcription factors in the pathology of MDD and stress. Considering that these transcription factors exert transcriptional control of the noradrenergic phenotypes, TH and DBH, the present studies may be linked to increased expression of TH in MDD (Zhu et al. 1999) and of TH and DBH in rodent stress models (Melia et al. 1992, Nankova et al. 1996, Fan et al. 2014).

Previous studies have revealed an apparent divergence in the distribution of Phox2a and Phox2b in animal brains. *Phox2a* is expressed in DBH-positive neurons such as A1, A2, A5 and the LC (A6) in postnatal mice (P12) (Tiveron et al 1996). However, the expression of *Phox2b* in the LC is transient and disappears around E11.5 rather than continuing up to birth like *Phox2a* (Pattyn et al 2000). Further, while a robust and stable expression of *Phox2a* can be found in all brainstem noradrenaline neurons of adult Sprague-Dawley rats (Card et al. 2010), there is no expression of *Phox2b* in the LC of the same strain of rats (Kang et al. 2007). Such divergent expression of *Phox2a* and *Phox2b* may indicate differences in their functions, and leads to the notion that expression of *Phox2b* is not necessary for the maintenance of the noradrenergic phenotype in the adult brainstem (Card et al. 2010). However, our previous study (Fan et al. 2011) and the current work showed that *Phox2b* is expressed in the LC of adult Fischer 344 rats. More importantly, gene expression and proteins of Phox2a and Phox2b are found in the LC of humans at ages ranging from 18–77 years (Table 1). These findings indicate that like *Phox2a*, *Phox2b* is expressed in the LC of adult humans and Fischer 344 rats, and may continue to play a significant role in the maintenance or modulation of noradrenergic transmission in normal and disease states.

Phox2a and Phox2b protein, as well as Phox2b gene expression, were significantly increased in the LC of MDD brain donors, compared to age-matched and psychiatrically normal control donors. This is the first report of the expression of Phox2a and Phox2b in the LC of humans that had MDD. It can be speculated that these genes may be involved in the neuronal control of adaptive homeostatic function in adulthood. Given psychiatric diseases are polygenetic disorders, aberrant gene expressions could be one of their pathophysiologic characteristics. Since transcription factors are involved in the orchestration of gene expression programs, abnormalities in their expression might be expected in MDD. Studies have shown that altered expression of certain transcription factors is associated with MDD. For example, CREB and phosphorylated CREB were found to be reduced in the temporal cortex of MDD patients (Yamada *et al.* 2003), resulting in an increased expression of pentraxin 3 (Shelton *et al.* 2004), transcription of the gene of which is inhibited by CREB (Sulser 2002). In addition, abnormal transcriptions caused by transcription factors LBP-1c (Schahab *et al.* 2006), Foxp2 (Li *et al.* 2013) and REST (Otsuki *et al.* 2010) have been associated with MDD. On the other hand, genetic disruption of Phox2 genes has been reported in other human diseases. For example, Phox2 gene mutations are associated with some human diseases such as the congenital fibrosis of the extraocular muscles syndrome type 2 (CFEOM2) (Nakano *et al.* 2001, Yazdani *et al.* 2003), congenital central hypoventilation syndrome (CCHS) (Amiel *et al.* 2003, Sasaki *et al.* 2003), and Hirschsprung disease (HSCR) (Carter *et al.* 2012, Fernandez *et al.* 2013). Considering the close relationship of *Phox2a* and *Phox2b* with the noradrenergic phenotype, an increased expression of Phox2a and Phox2b in the LC of MDD may suggest that these transcription factors could be a molecular marker for the pathophysiology of MDD. Further exploration of their roles in MDD might provide additional information for their genetic linkage with MDD.

Stress is a major risk factor for the development of depression, and stress induces release of CORT (in rodents) or cortisol (in humans) (Chrousos & Gold 1992). Hence, it is noteworthy that relatively similar enhancements of Phox2a and Phox2b were observed in the LC in MDD donors and Fischer 344 rats exposed to chronic stress, although CORT treatments only resulted in an increase of Phox2b in rats. It is conceivable that the molecular mechanisms responsible for elevated Phox2a and Phox2b in MDD and chronically stressed rats are similar. At least for Phox2b, the mechanism may involve CORT in rats, or cortisol in humans. Interestingly, several stress paradigms including single and repeated immobilization elicited expression levels of c-Fos, ΔFosB, Fra-2 and CREB in the LC (Hebert *et al.* 2005, Goebel *et al.* 2009, McDevitt *et al.* 2009, Imbe & Kimura 2016), as well as c-Fos, Fra-2, Egr1 in other brain regions such as the amygdala, as well as in the adrenal medulla (Sabban *et al.* 2004, Porter & Hayward 2011). Also of note is that *Phox2b* expression levels were significantly increased in adrenal medulla of stressed mice after 7 days of unpredictable chronic stress (Santana et al. 2015). Therefore, the results of the present study are consistent with these previous investigations.

One issue raised by the present study is physiological relevance of the upregulated Phox2a and Phox2b in the LC of MDD and stressed animals. Given *Phox2a* and *Phox2b* are essential for development of the noradrenergic system in the embryonic period, their upregulation may be related to their effects on the noradrenergic phenotype in adulthood too.

Postmortem studies demonstrated biological abnormalities of the LC, including altered TH protein levels in subjects who suffered from MDD (Zhu et al. 1999) or committed suicide (Ordway et al. 1994, Xiang *et al.* 2004). Animal studies demonstrate that chronic stress significantly increases TH protein in the LC (Melia & Duman 1991, Smith *et al.* 1991, Brady 1994, Watanabe *et al.* 1995, McDougall *et al.* 2005). Studies from our and other laboratories report a marked elevation of DBH expression in the LC of stressed animals (Serova *et al.* 1999, Fan *et al.* 2013). In addition, stress-induced DBH upregulation was found in the stellate ganglia (Gavrilovic *et al.* 2009), adrenal medulla (Nankova *et al.* 1999, Spasojevic *et al.* 2010) and sympathetic ganglia (Kvetnansky *et al.* 2004), where *Phox2a* and *Phox2b* are normally expressed (Brunet & Pattyn 2002). It seems likely that induction of *Phox2a* and *Phox2b* expression may be a precursor to stress-induced in increases in LC activity and *TH* expression. Therefore, an increased expression of *Phox2a* and *Phox2b* would account for the upregulated expression of TH and DBH in the LC under depressive and stressful conditions. Although we do not have ample direct evidence to support this explanation currently, our previous study showed that forced expression of *Phox2a* and *Phox2b* in the LC favors the increased expression of the noradrenergic phenotype in adult rats (Fan et al. 2011). More studies are necessary to clarify this important issue.

In this study, compared to CSD paradigm, chronic administration of CORT to rats did not significantly upregulate *Phox2a* or Phox2a protein expression. This unchanged Phox2a expression might not be ascribed to CORT administration, as the *in vitro* study showed mRNA and protein levels of Phox2a and Phox2b were upregulated by exposure of cells to CORT. The possible reason for the discrepancy may be related to physiological or pharmacokinetic differences between the stress paradigm and oral CORT administration. This is similar to the observation in our previous study that while CSD upregulated DBH protein levels in all major terminal regions including the frontal cortex (Fan et al. 2013), but there was no significant alteration of DBH protein levels in the frontal cortex after CORT ingestion (Fan et al. 2014). Investigations from other laboratories also demonstrate discrepant results between stress paradigms and CORT treatments. For example, chronic stress significantly reduced expression of brain-derived neurotrophic factor (BDNF) in the frontal cortex (Roceri *et al.* 2004, Mao *et al.* 2010, Li *et al.* 2012), whereas chronic administration of CORT had no significant effect on *BDNF* mRNA or BDNF protein levels in the same brain region (Jacobsen & Mork 2006). The discrepancy in Phox2a expression effects between CSD paradigm and CORT ingestion may also be related to the fact that stress can activate multiple hormones and signaling pathways and therefore produce more complex effects than exposure to CORT alone.

The present study demonstrated intriguing findings regarding the alterations of Phox2 expression in MDD and induced by stress in rats. However, it is noteworthy that Phox2a mRNA levels in the LC from MDD donors, and mRNA and protein levels of Phox2a in the LC of rats exposed to CORT did not exhibit the significant changes as seen for Phox2b. One potential limitation for such complete correspondence between Phox2a and Phox2b may be a result of small sample sizes that precluded the demonstration of statistical significance. Small sample sizes reduced the power of the study and increased the margin of error. It will therefore be necessary to replicate our finding, especially for the human tissues, with a larger sample size.

In conclusion, the present study demonstrated that transcription factors Phox2a and Phox2b are expressed in the LC of adult humans and Fischer 344 rats, and the expression of these transcription factors appear to be plastic. *In vitro* experiments demonstrated that the expression of both Phox2a and Phox2b are upregulated after exposure of cells to CORT. Furthermore, CORT-induced upregulation of Phox2b is mediated by corticosteroid receptors, as verified by *in vivo* and *in vitro* experiments using corticosteroid receptor antagonists. Taken as a whole, the results of this study strongly suggest that Phox2a and Phox2b may play a regulatory role in the mature brain. Their altered expression in MDD and following chronic stress suggest that these transcription factors may contribute to neuronal pathology associated with these conditions.

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Abbreviations:

CORT:	corticosterone
CSD:	chronic social defeat
DBH:	dopamine βhydroxylase
ir:	immunoreactivity
LC:	locus coeruleus
MDD:	major depressive disorders
PBS:	phosphate-buffer saline
q-PCR:	quantitative real-time polymerase chain reaction
TH:	tyrosine hydroxylase

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Highlights

- Protein levels of Phox2a, mRNA and protein levels of Phox2b were elevated in the locus coeruleus from brain donors of MDD
- CSD significantly increased Phox2a and Phox2b expression in the rat locus coeruleus
- Corticosterone administration increased Phox2b protein levels in the rat locus coeruleus
- Corticosterone increased Phox2a and Phox2b expression in the SK-SY5Y cells
- Corticosterone-induced increase in Phox2 expression may be mediated through corticosteroid receptors

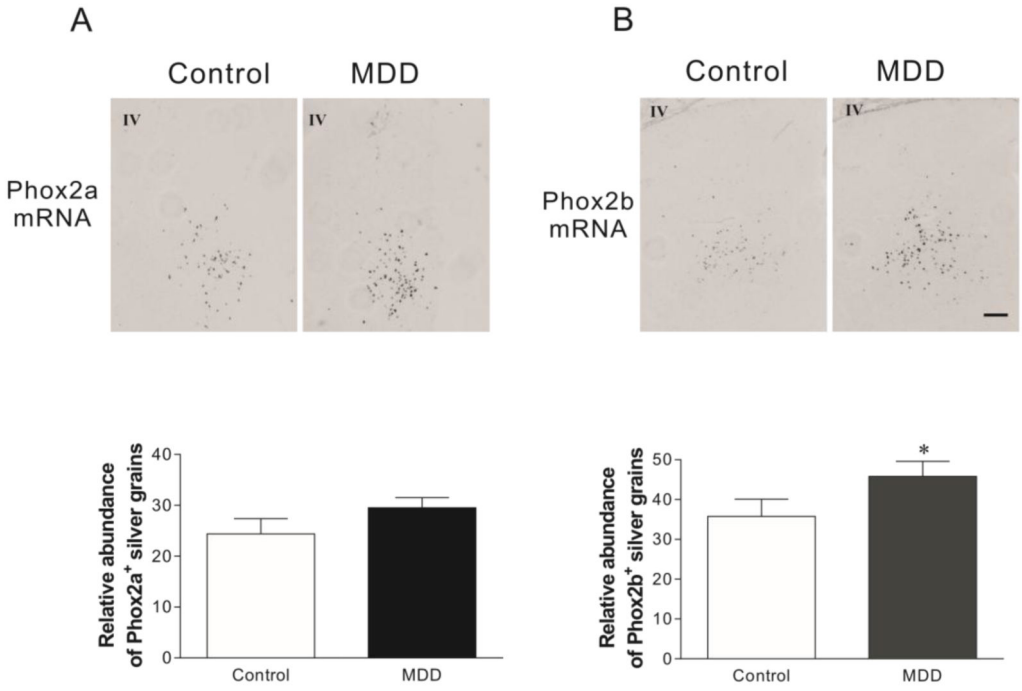

Figure 1:

The expression of Phox2a (A) and Phox2b (B) mRNAs in the LC of MDD donors and age-matched and psychiatrically normal control donors (N=12 for both). *Upper panel:* Representative images of Phox2a and Phox2b mRNA in the LC of brains detected by *in situ* hybridization. *Lower panel:* Quantitative analyses of Phox2a and Phox2b mRNA hybridizations for control and MDD donors. IV: Fourth ventricle. Scale bar: 250 μm for all images.

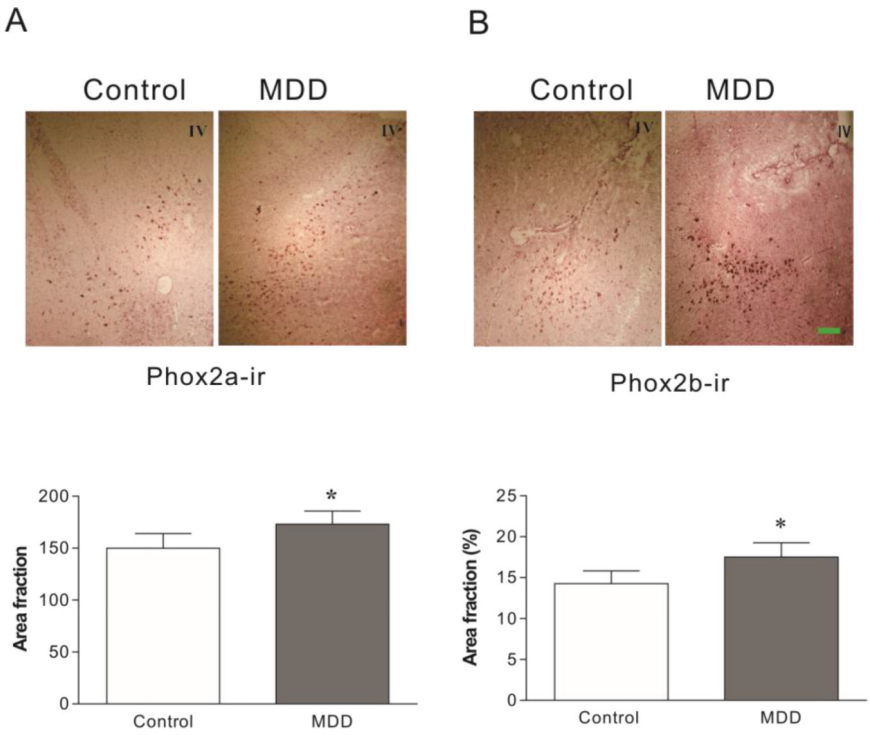

Figure 2:

Phox2a and Phox2b immunoreactivity (ir) in the LC of MDD donors and age-matched psychiatrically normal control donors (N=12 for both). *Upper panel:* Representative images of Phox2a- and Phox2b-ir in the LC of brains detected by immunocytochemical staining. *Lower panel:* semi-quantitative analyses of Phox2a-ir and Phox2b-ir in tissue sections. * $p<0.05$, compared to the control. IV: Fourth ventricle. Scale bar: 250 μm for all images.

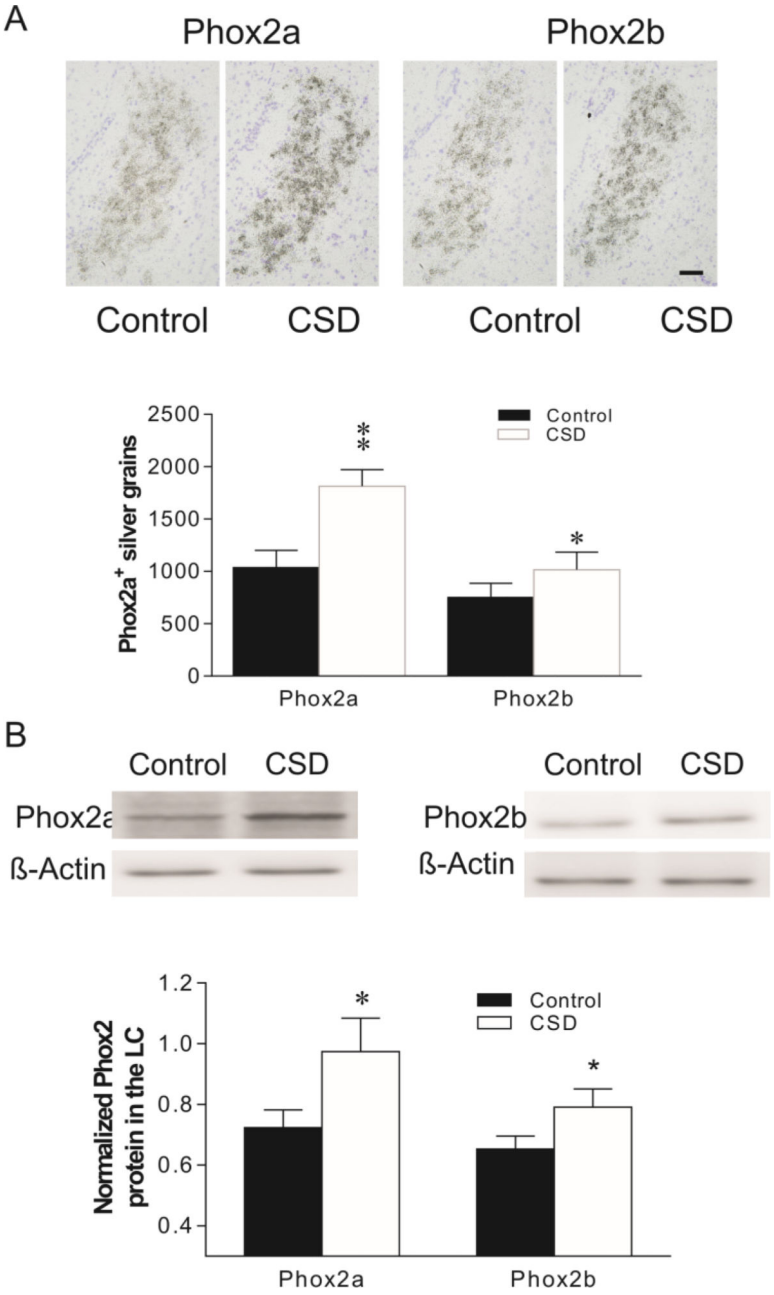

Figure 3:

Effects of CSD on mRNAs (A) and protein (B) levels of Phox2a and Phox2b in the rat LC. *Upper panel in A:* Phox2a/2b mRNA in LC of rat brains detected by *in situ* hybridization (N=7/group). *Lower panel in A:* Quantitative analyses of mRNA in slides. *Upper panel in B:* Autoradiographs obtained by western blotting of Phox2a/2b in LC of rat brains (N=8/group). *Lower panel in B:* Quantitative analyses of band densities. Values of Phox2 bands were normalized to those of β-actin probed on the same blot. * $p<0.05$, ** $p<0.01$, compared to the control group. Scale bar: 50 μm for all images.

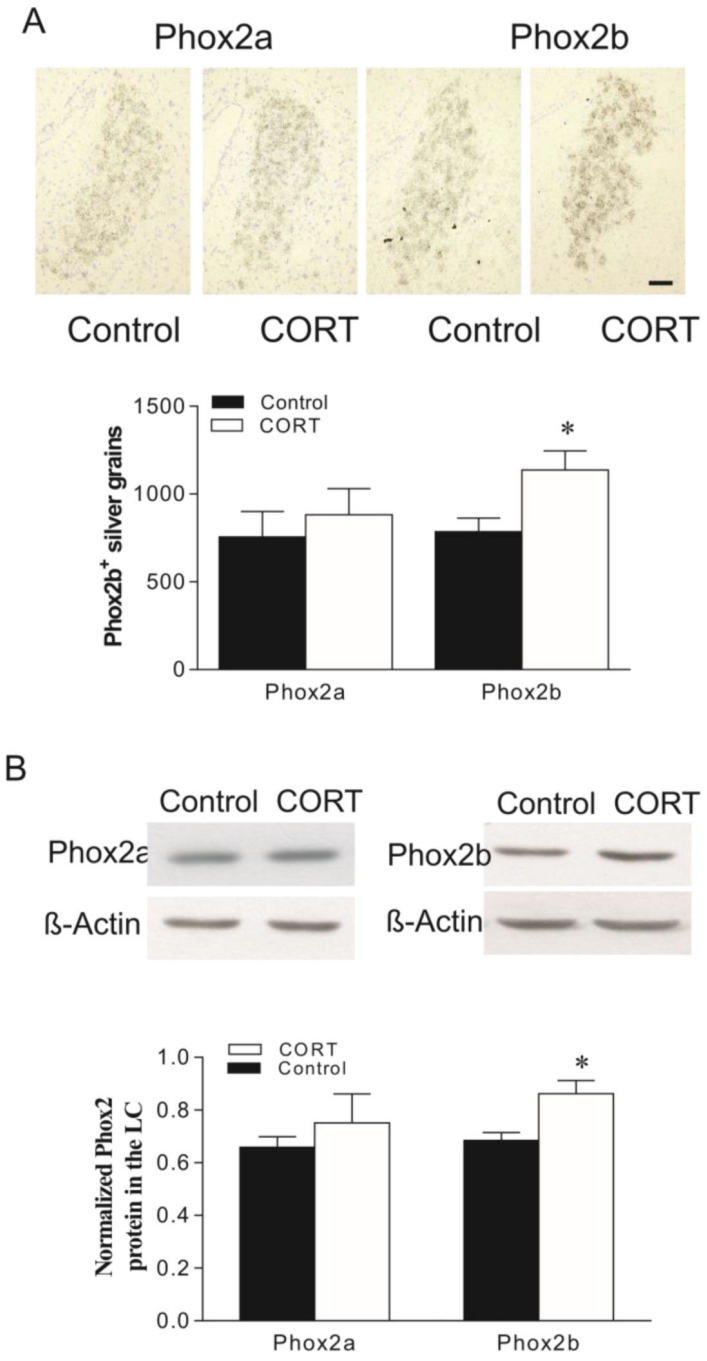

Figure 4:

Effects of oral administration of CORT on mRNAs (A) and protein (B) levels of Phox2a and Phox2b in the rat LC. *Upper panel in A:* Phox2a/2b mRNA in LC of rat brains detected by *in situ* hybridization (N=7/group). *Lower panel in A:* Quantitative analyses of mRNA in slides. *Upper panel in B:* Autoradiographs obtained by western blotting of Phox2a/2b in LC of rat brains (N=8/group). *Lower panel in B:* Quantitative analyses of band densities. Values of Phox2 bands were normalized to those of β-actin probed on the same blot. * $p<0.05$, compared to the control. Scale bar: 50 μm for all images.

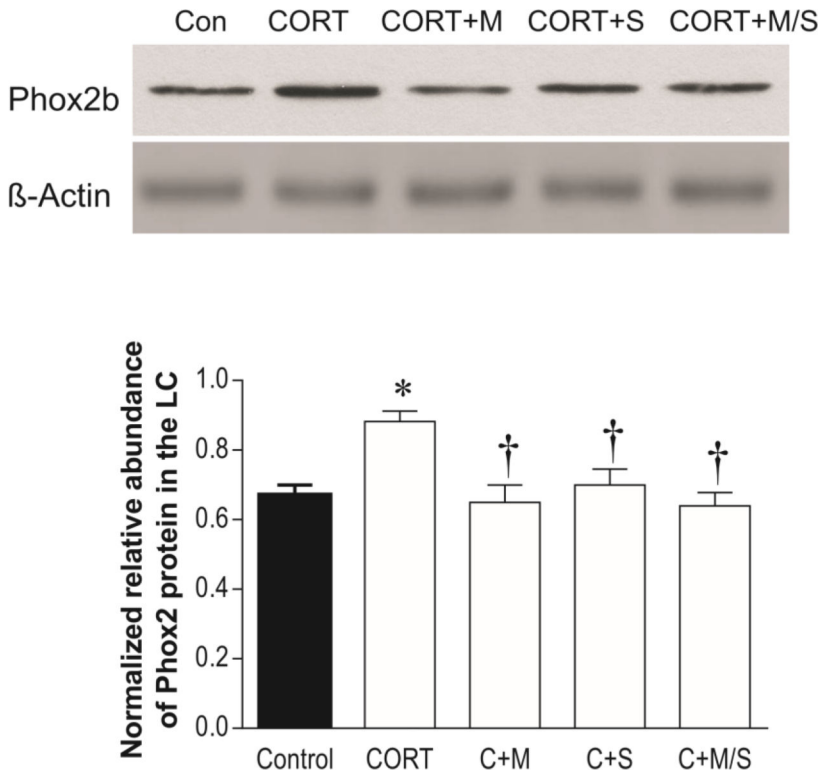

Figure 5:

Effects of CORT administration, and CORT administration plus treatment with corticosteroid receptor antagonists (N=7/group) on Phox2b immunoreactivities in the LC. *Upper panel*: Phox2b proteins in LC tissues of rats detected by western blotting. *Lower panel*: Quantitative analyses of band densities. * $p<0.05$, compared to the control; † $p<0.05$, compared to the CORT group. Con: control; CORT+M: CORT plus treatment with mifepristone; CORT+S: CORT plus treatment with spironolactone; C+M/S: CORT plus treatments with both mifepristone and spironolactone.

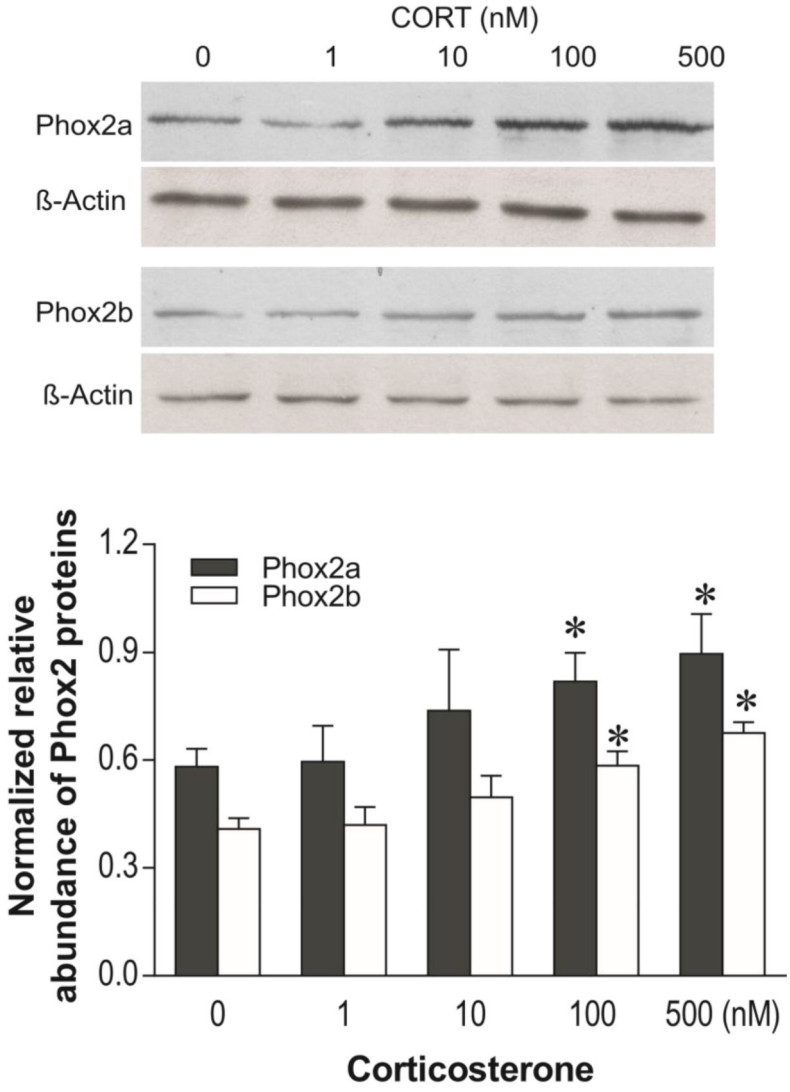

Figure 6:

Effects of exposing SH-SY5Y cells to different concentrations of CORT for 3 days on immunoreactivities of Phox2a and Phox2b (N=5/groups). *Upper panel*: Phox2a and Phox2b proteins detected by western blotting. *Lower panel*: Quantitative analyses of band densities. * $p<0.05$, compared to the 0 group (vehicle).

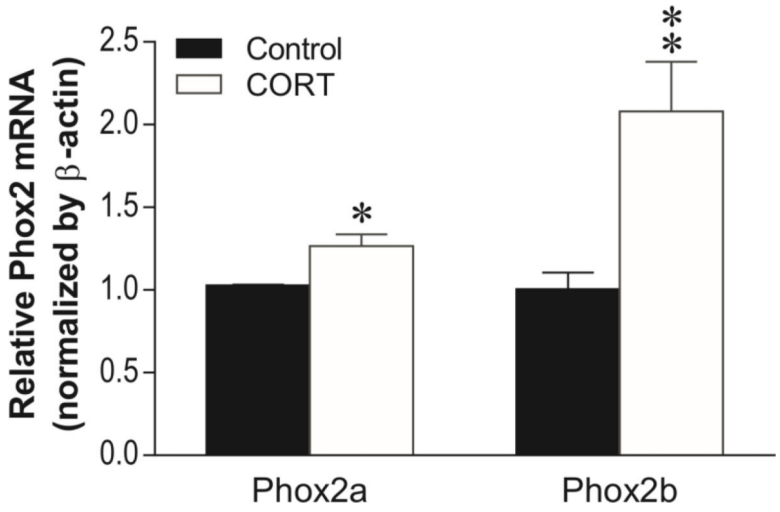

Figure 7:

Effects of exposing to CORT on mRNA levels of Phox2a and Phox2b. SH-SY5Y cells were exposed to 100 nM CORT for 3 days. Harvested cells were prepared for real-time PCR with β-actin as the internal control. The graphic data represent means ± SEM obtained from 7 separate experiments (N=7). *$p < 0.05$, ** $p < 0.01$; compared to the group treated with vehicle (control).

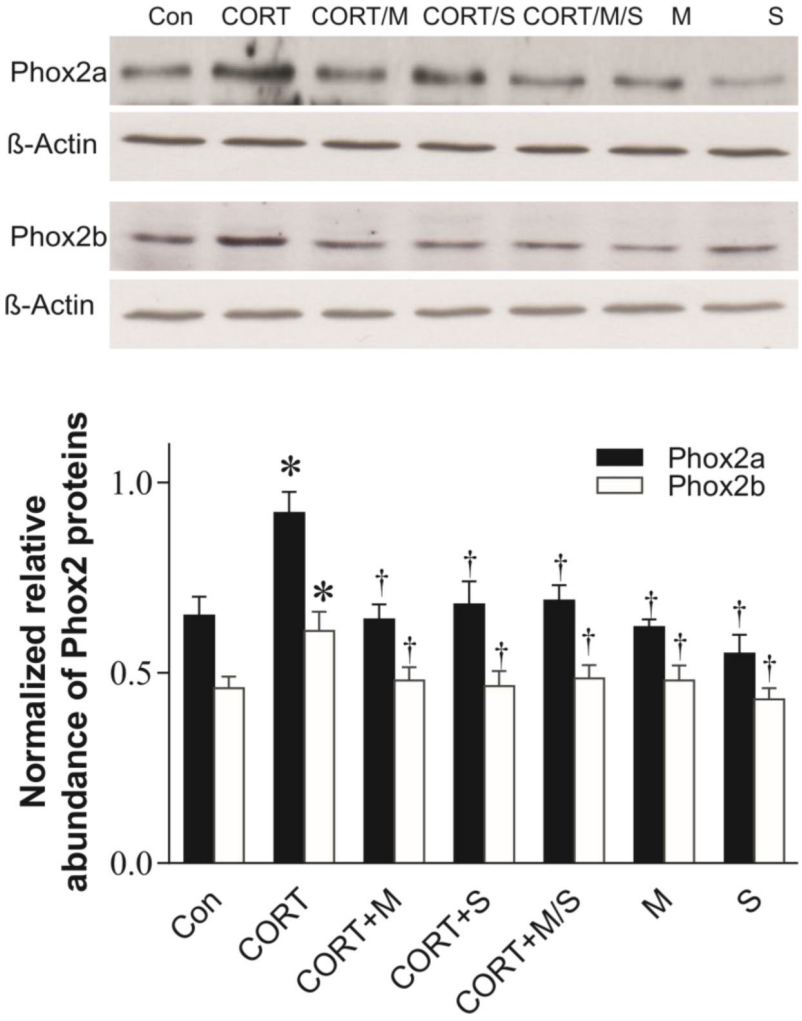

Figure 8:

Effects of CORT receptor antagonists on CORT-induced increases in Phox2b immunoreactivities. SH-SY5Y cells were simultaneously exposed to 100 nM CORT, CORT plus either mifepristone, spironolactone or both, as well as mifepristone, spironolactone alone for 3 days (N=5/group). *Upper panel*: Phox2b proteins detected by western blotting. *Lower panel*: Quantitative analyses of band densities. * $p<0.05$, compared to the control (Con), † $p<0.05$, compared to the CORT ingestion group. M: treated with mifepristone alone; S: treated with spironolactone; for other abbreviations see legend of Fig. 6.

Table 1.

Subject demographics

Subject Code	pH	RIN[a]	PMI[b]	Smoker	Toxicology	Axis I diagnosis	Suicide	Tissue	Cause of death
Normal Control Donors									
KS 59	6.95	6.8	19.0	No	Ethanol (0.03%)	None	No	LC	Heart disease
KS 31	6.79	7.6	6.0	No,hx[d]	Lidocaine	None	No	LC	Heart disease
KS 63	6.40	6.4	31.5	unknown	Midazolam	None	No	LC	Btonchial asthma
KS 65	5.80	6.4	27	No	NDD[c]	None	No	LC	Accident
KS 82	6.50	6.5	26	No	Morphine	None	No	LC	Paritonitis
KS 70	7	7.4	22.3	No	NDD	None	No	LC	Distended aorta
KS 76	6.70	7.4	23.5	No	Butalbital	None	No	LC	Trauma
KS 78	6.60	7.9	16.0	unknown	NDD	None	No	LC	Cong. heart[e]
KS 74	6.30	7.3	16.4	No	Diltiazem	None	No	LC	Trauma
KS 85	6.60	7	24.9	No	Diltiazem	None	No	LC	Trauma
KS 23	6.78	7.7	21	Yes	NDD	None	No	LC	Heart disease
KS 72	7.10	8.5	19.9	Yes	Doxylamine	None	No	LC	Subarachnoid hemorrhage
MEAN	**6.63**	**7.24**	**21.15**						
SEM	0.10	**0.18**	**1.89**						
MDD Donors									
KS 56	6.47	6.9	31	No	Ethanol	MDD	No	LC	Gunshot
KS 32	6.32	6.8	20	Yes	Ethanol	MDD	No	LC	Gunshot
KS 66	6.68	6.7	17	No	NDD	MDD	No	LC	Asphyxiation
KS 55	6.42	7.4	11.0	No	NDD	MDD	No	LC	Heart disease
DD	6.48	5.8	18	No	CO	MDD	No	LC	CO poisoning
KS 71	6.60	7.5	5.3	No	Citalopram, Venlafaxine	MDD	No	LC	Hanging
KS 77	6.70	8.7	18.8	No	NDD	MDD	No	LC	Gunshot
KS 79	6.90	7.6	12.9	Yes	NDD	MDD	No	LC	Gunshot
KS 75	6.70	6.7	19.6	No	CO, Diazepam, Temazepam	MDD	No	LC	CO poisoning
KS 81	7	8.5	10.1	No	Ethanol	MDD	No	LC	Hanging
KS 24	6.85	7.25	26	Yes	Ethanol	MDD	No	LC	Gunshot
KS 73	6.50	9.2	11	Yes	NDD	MDD		LC	Heart disease
MEAN	**6.64**	**7.42**	**16.73**						
SEM	**0.06**	**0.28**	**2.08**						

[a] RNA integrity number generated by the Agilent Bioanalyzer 2100®

[b] Postmortem interval

[c] No drugs detectable

[d] History

[e] Congestive heart failure

Table 2.

Pearson correlations for potential confounding variables

		mRNA		Protein	
		Phox2a	**Phox2b**	**Phox2a**	**Phox2b**
pH	Pearson Correlation	−0.249	−0.286	−0.017	0.242
	Sig. (2-tailed)	0.241	0.176	0.938	0.255
	N	24	24	24	24
PMI	Pearson Correlation	−0.047	−0.366	0.105	−0.187
	Sig. (2-tailed)	0.826	0.078	0.624	0.382
	N	24	24	24	24
RIN	Pearson Correlation	0.154	−0.076	−0.035	0.193
	Sig. (2-tailed)	0.471	0.725	0.87	0.367
	N	24	24	24	24
Age	Pearson Correlation	0.263	−0.035	0.001	0.434
	Sig. (2-tailed)	0.213	0.869	0.998	0.034
	N	24	24	24	24