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Effect of methylphenidate treatment during adolescence on norepinephrine transporter function in orbitofrontal cortex in a rat model of Attention Deficit Hyperactivity Disorder

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Abstract

Attention Deficit Hyperactivity Disorder (ADHD) is associated with hypofunctional medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC). Methylphenidate (MPH) remediates ADHD, in part, by inhibiting the norepinephrine transporter (NET). MPH also reduces ADHD-like symptoms in Spontaneously Hypertensive Rats (SHRs), a model of ADHD. However, effects of chronic MPH treatment on NET function in mPFC and OFC in SHR have not been reported. In the current study, long-term effects of repeated treatment with a therapeutically relevant oral dose of MPH during adolescence on NET function in subregions of mPFC (cingulate gyrus, prelimbic cortex and infralimbic cortex) and in the OFC of adult SHR, Wistar-Kyoto (WKY, inbred control) and Wistar (WIS, outbred control) rats were determined using in vivo voltammetry. Following local ejection of norepinephrine (NE), uptake rate was determined as peak amplitude (A_{max}) x first-order rate constant (k_{-1}). In mPFC subregions, no strain or treatment effects were found in NE uptake rate. In OFC, NE uptake rate in vehicle-treated adult SHR was greater than in adult WKY and WIS administered vehicle. MPH treatment during adolescence normalized NE uptake rate in OFC in SHR. Thus, the current study implicates increased NET function in OFC as an underlying mechanism for reduced noradrenergic transmission in OFC, and consequently, the behavioral deficits associated with ADHD. MPH treatment during adolescence normalized NET function in OFC in adulthood, suggesting that the therapeutic action of MPH persists long after treatment cessation and may contribute to lasting reductions in deficits associated with ADHD.

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Keywords

1 Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most prevalent neurobehavioral disorders, affecting ~12–15% of children worldwide and persisting into adulthood in ~4–5% of individuals (Froehlich et al., 2007; Polanczyk et al., 2007). ADHD etiology, although multigenetic (Faraone et al., 2005; Gizer et al., 2009; Kuntsi and Klein, 2012), has been associated with polymorphisms of the norepinephrine transporter (NET) gene (Barr et al., 2002; Kollins et al., 2008; McEvoy et al., 2002). However, altered noradrenergic function in ADHD has not been verified in clinical studies (Zimmer, 2009), in part, due to the paucity of suitable radioligands probing NET for use in positron emission tomography studies (Ding et al., 2006). Importantly, compelling evidence supporting the involvement of the noradrenergic system in ADHD pathology comes from the common mechanism of action of all of the currently available FDA-approved therapeutics for ADHD. ADHD medications increase noradrenergic neurotransmission either via inhibition of NET function (e.g., methylphenidate; MPH, amphetamine and atomoxetine) or by activation of post-synaptic α2A adrenergic receptors (e.g., guanfacine) (Berridge et al., 2006; Bymaster et al., 2002; Fernando et al., 2012; Ma et al., 2005). Thus, ADHD pathology is strongly connected to deficits in noradrenergic neurotransmission and the efficacy of ADHD therapeutics is associated with increased noradrenergic transmission (Levy, 2009).

The prefrontal cortex has been associated with the hallmark symptoms of ADHD and has been linked to the beneficial effects of ADHD therapeutics in preclinical models (Arnsten, 2009; Berridge and Devilbiss, 2011). For example, administration of a therapeutically relevant low dose of MPH (2.0 mg/kg, i.p.) in rats produced positive blood oxygen dependent signals in the orbitofrontal cortex (OFC), a subregion of prefrontal cortex (Easton et al., 2009; Kuczenski and Segal, 2002). Inhibition of α2 adrenergic receptors by local administration of yohimbine into the dorsolateral prefrontal cortex of primates resulted in impaired spatial working memory, increased impulsivity and increased locomotor hyperactivity, thus resulting in behaviors consistent with an ADHD profile (Li and Mei, 1994; Ma et al., 2005; Ma et al., 2003) and suggesting a role for noradrenergic neurotransmission in prefrontal cortex in ADHD pathology.

Effects of NET inhibition by ADHD therapeutics on prefrontal cortex function have been elaborated also using preclinical models. MPH is an equipotent inhibitor of NET and dopamine transporter (DAT), with Ki values for inhibition of [^{3}H]dopamine and [^{3}H]norepinephrine (NE) uptake of 160 nM and 40 nM, respectively (Easton et al., 2007b; Richelson and Pfenning, 1984). At therapeutically relevant doses (0.5 - 2 mg/kg, i.p.), MPH inhibits NET function and preferentially increases extracellular NE in prefrontal cortex compared to subcortical areas, as well as compared to dopamine in prefrontal cortex (Berridge et al., 2006). Increasing extracellular NE activates α2A receptors and decreases the concentrations of the intracellular second messenger, cyclic adenosine monophosphate

(cAMP), thereby enhancing the strength and duration of firing of pyramidal neurons in prefrontal cortex (Wang et al., 2007). Enhanced pyramidal neuronal firing is hypothesized to be the mechanism by which ADHD medications improve attention and working memory and reduce impulsivity (Sagvolden, 2006; Wang et al., 2007). MPH indirectly increases the activation of cortical α2A adrenergic receptors in non-human primates and rats (Arnsten, 2009; Gamo et al., 2010). Thus, enhancing noradrenergic neurotransmission in the prefrontal cortex via inhibition of NET function is critical for the therapeutic action of MPH. However, few studies have investigated the long-term consequences of MPH treatment during adolescence on NET function during adulthood.

The Spontaneously Hypertensive Rat (SHR) is a well-established model of ADHD that exhibits several of the behavioral and neurochemical features of ADHD (de Villiers et al., 1995; Oades et al., 2005; Russell et al., 1995; Sagvolden, 2011; Sagvolden et al., 2005). In SHR, noradrenergic systems in the locus coeruleus and hypothalamus are inadequately regulated by α2A autoreceptors and heterologous glutamatergic receptors, resulting in enhanced NE release (de Villiers et al., 1995; Howells and Russell, 2008; Russell et al., 2005). Importantly, α2A auto-receptor mediated regulation of NE release is decreased in prefrontal cortical slices from SHR (Russell et al., 2000). Notably, alterations in NET function in the prefrontal cortex of SHR have not been reported.

Previous studies in SHR found that MPH treatment during adolescence produces lasting changes in DAT function in prefrontal cortex as well as in its subregion, the medial prefrontal cortex (mPFC; Harvey et al., 2011; Somkuwar et al., 2013). Specifically, DAT function was increased in mPFC of MPH-treated SHR compared to vehicle-treated SHR as well as compared to MPH-treated Wistar Kyoto rats (WKY, inbred control) and Wistar rats (WIS, outbred control). The aim of the current study was to evaluate *in vivo* NET function in subregions of prefrontal cortex, including the cingulate gyrus (CG), prelimbic cortex (PrL), infralimbic cortex (IL) and lateral orbitofrontal cortex (LO). We chose to separately target the CG, PrL and the IL cortex subregions of mPFC, because these regions have distinct intracortical and subcortical connections (Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007; Vertes, 2006). Specifically, the CG and the dorsal PrL are thought to project to motor and sensory cortices, while the ventral PrL and IL project to limbic and associative areas. As such, these individual subregions may differentially influence ADHD pathology (Easton et al., 2007a). Also, the current study describes a novel method for isolating NET function in prefrontal cortex using an *in vivo* voltammetry approach. Differences were revealed between SHR and the WKY and WIS controls following administration of MPH or vehicle throughout adolescence on NET function during adulthood.

2 Materials and Methods

2.1 Drugs and Reagents

(±)-MPH was purchased from Sigma-Aldrich Corporation (St. Louis, MO). MPH was dissolved in water to obtain a concentration of 1.5 mg/ml (prepared daily) and injected into oyster crackers (Kantak et al., 2008) to attain a dose of 1.5 mg/kg for oral administration. (±)-Norepinephrine (+)-bitartarate (NE), GBR12909 (1-2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride), ascorbic acid,

perchloric acid (70%), sodium chloride and Nafion perfluorinated ion-exchange resin (5% solution) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Sodium phosphate dibasic and monobasic were purchased from Fischer Scientific (Fair Lawn, NJ). Urethane and sticky wax were purchased from Sigma Life-Sciences (St. Louis, MO) and FDJ/On time LLC (Winter Park, FL), respectively.

2.2 Animals

For optimization of the NE clearance assay, male and female adult Sprague-Dawley rats were obtained from the Harlan Laboratories Inc. (Indianapolis, IN). Some of the rats used for optimization experiments were drug and experimentally naïve and others experienced operant or Pavlovian conditioning, including acute treatment with morphine (10 mg/kg or 45 mg/kg, i.p.). Consequently, rats that underwent conditioning experienced greater handling and enrichment compared to the experimentally naïve rats. Although optimization experiments to identify the effect of DAT inhibition on NE clearance in PFC were conducted 1–2 weeks after treatment, large variability in the data may have been a consequence of the variation in treatment history.

For experiments investigating the effect of MPH treatment during adolescence, naive male SHR, WKY and WIS rats were obtained at postnatal day (P) 25 from Charles River Laboratories/US facilities (Kingston, NY or Raleigh, NC). Vendor location is a critical to ensure homogeneity with respect to ADHD (SHR) and non-ADHD (WKY and WIS) phenotypes. WKY from Charles River/GER have been characterized as a model for the predominantly inattentive subtype of ADHD (Sagvolden et al., 2009), and have a large number of discordant SNP genotypes (35%) relative to WKY from Charles River/US and Harlan/UK, which have only 2.5% discordant SNP genotypes (Zhang-James et al., 2013). Further, WKY from Harlan/UK is a well-recognized control for the SHR ADHD phenotype (Sagvolden et al., 2009), supporting the use of WKY from Charles River/US as the inbred control for the current studies.

All rats were individually housed with free access to food and water (unless specified otherwise) in a colony room maintained on a 12-hour light:dark cycle (lights on 07:00 h) in the DLAR (University of Kentucky, Lexington, KY). All experimental and rat handling procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and were performed in accordance with the 1996 version of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

2.3 Treatment

Following three days of habituation, SHR, WKY and WIS rats were administered MPH (1.5 mg/kg, p.o.) or vehicle (VEH, water, 1 ml/kg, p.o.) from P28 to P55, the time period from early to late adolescence (Doremus-Fitzwater et al., 2010; Spear, 2000). MPH was administered orally to mimic the route of administration employed in patients with ADHD. MPH or vehicle was administered daily Monday through Friday to mimic the weekend medication holiday often practiced in the treatment of children and adolescents with ADHD (American Psychiatric Association., 2013). The dose of MPH (1.5 mg/kg, p.o.) in outbred rats provides clinically relevant plasma concentrations of MPH (Wargin et al., 1983) that

increase extracellular NE and dopamine in prefrontal cortex, but are below threshold for producing locomotor activation or for increasing dopamine concentrations in striatum (Berridge et al., 2006; Kuczenski and Segal, 2002). In adolescent SHR, MPH (1.5 mg/kg, p.o.) improves attention and behavioral flexibility when administered from P28-P55 (Harvey et al., 2013; Harvey et al., 2011).

From P56 onwards, rats were housed individually with free access to food and water in a colony room until the electrochemical experiments were conducted. Between P77-91 rats were anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Urethane was chosen because it does not alter DAT function *in vivo* (Sabeti et al., 2003). Body temperature was maintained at 34–35°C (rat core temperature; DeBow and Colbourne, 2003) using a heating pad coupled with a rectal thermometer (Harvard Apparatus, Holliston, MA). A longitudinal incision was made in the scalp and the skin was retracted. Bleeding was stopped by local application of epinephrine (5 μg/ml, in saline) and a styptic-powder with benzocaine (Kwikstop, ARC Laboratories, Atlanta, GA). Excess epinephrine was washed away and the exposed skull was air-dried. A small hole was drilled into the skull over the posterior cortex for placement of an Ag/AgCl silver reference electrode. A larger hole was drilled into the skull overlying the mPFC and LO, and the dura was removed to expose the cortex. The exposed cortex was cleaned of blood using sterile saline. The electrode-micropipette assembly was lowered into the CG (+ 2.9mm AP, ± 0.8 mm ML, -1.6 mm DV), PrL (dorsal +2.9 mm AP, ± 0.8 mm ML, -2.5 mm DV and ventral +2.9 mm AP, ± 0.8 mm ML, -3.2 mm DV), IL (+2.9 mm AP, ± 0.8 mm ML, -4.2 mm DV) and LO (+3.2 mm AP, ± 2.6 mm ML, -4.2 mm DV). Typically, recordings from the mPFC subregions and from the LO were conducted in the contralateral hemispheres (randomized between experiments) using a single electrode-micropipette assembly for recording from all the sites. However, if the assembly failed during any portion of the experiments (e.g., due to technical issues such as pipette clogging, loss in electrode sensitivity, etc.), a fresh calibrated electrode-micropipette assembly was used. In such cases, electrochemical recordings were conducted subsequently from the opposite hemisphere to avoid the subregion that may have been damaged by the previous electrode-micropipette assembly.

2.4 *In vivo* electrochemical measurements

High speed chronoamperometry is an electrochemical method for detecting molecules that readily undergo oxidation and reduction when in contact with an electrode held at an appropriate voltage; concentration of the molecule is directly proportional to the oxidation and reduction currents (Borland and Michael, 2007). NE and dopamine are electroactive analytes in which the catechol ring readily oxidizes to an orthoquinone and then reduces back to the catechol ring when a 0 V to 0.55 V square-wave pulse is applied in a physiological (pH 7.4) buffer. Specific DAT function has been inferred from real-time clearance of exogenously applied dopamine, thereby circumventing the potential confound of endogenous dopamine released from presynaptic terminals altering clearance parameter measurements (McCrae et al., 2005). DAT and NET share functional and structural homologies and have comparable substrate selectivity (Miller et al., 2010; Moron et al., 2002; Whiteside et al., 2005). Hence, the electrochemical procedures for NET function

employed in the current study were derived from methods from previous studies evaluating DAT function (Liu et al., 2009; Zhu et al., 2007). To assess NET function, NE clearance from the extracellular space was determined following local ejection of NE into Cg, PrL, IL and LO in urethane anesthetized rats based on published methods for evaluation of dopamine clearance (Zhu et al., 2007) with a few modifications. Briefly, the electrochemical recording electrodes (Center for Microelectrode Technology, Lexington, KY) consisted of a single carbon fiber (30 μm diameter) sealed in a glass capillary with 150 to 200 μm of the fiber length exposed. To enhance electrode selectivity for positively-charged catecholamines over negatively-charged endogenous analytes (e.g., ascorbic acid), the exposed region of the carbon-fiber electrode was coated with Nafion (10 swirls) and cured by heating at 200 °C for 5 min (Gerhardt et al., 1984). Electrodes were calibrated in vitro in 0.1 M phosphate buffer saline (pH 7.4) at room temperature and showed a linear current by concentration response for NE (2–8 μM) with correlation coefficients ranging from 0.98 to 1.00. A Fast16 mkIII system (Fast Analytical Sensing Technology, Quanteon, LLC, Nicholasville, KY) was used for electrode calibration and subsequent *in vivo* experiments. Selectivity for NE over ascorbic acid ranged from 30 - 6800 to 1, which results in an acceptable 3.1% variation (range 96.7 – 99.8%) between the most and the least accurate NE signals. Electrode selectivity depends on environmental temperature and humidity during the Nafion coating process. The process was repeated until selectivity for NE over ascorbic acid reached >30:1. The limit of detection for the electrodes ranged from 0.01 – 0.51 μM (average = 0.11 μM). Each electrode was attached to a single-barrel micropipette (internal tip diameter, 10 – 20 μm) with sticky-wax such that the electrode and the micropipette were 100–400 μm apart.

For optimization experiments determining NET function in the absence and presence of GBR12909, the micropipette, immediately prior to lowering the recording assembly into the region of interest, was filled with a non-saturating NE solution (200 μM) with ascorbic acid (100 μM) in saline (0.9% sodium chloride; Hospira Inc, IL) at pH 7.2–7.4. The selected concentration of NE (200 μM) was based on prior studies evaluating dopamine clearance in the mPFC using *in vivo* voltammetry (Zhu et al., 2007). Although, NET expression is greater than DAT in prefrontal cortex, NE has a high affinity for DAT, and thus, NE may be nonspecifically cleared by DAT (Moron et al., 2002). Clearance of NE by DAT was inhibited by including the specific DAT inhibitor GBR12909 (Andersen, 1989) to isolate NET function. GBR12909 (50 nM) was used at a concentration ~15-fold higher than the Ki for inhibition of dopamine uptake at DAT (Matecka et al., 1997). Effects of DAT inhibition on NE clearance in mPFC subregions and in LO were evaluated using a within-subject design (n = 3–4/group). Brain regions of interest in one hemisphere received NE solution (200 μM) containing GBR12909 (50 nM) and the other hemisphere received NE solution in the absence of GBR12909, with conditions randomized between experiments. At 5 min intervals, the NE solution in the absence or presence of GBR12909 was pressure-ejected (20–25 psi) from the micropipette using a Picospritzer II (General Valve Corporation, Fairfield, NJ). The volume ejected (~264 nl/mm) was measured using a reticule with a millimeter scale fitted in the eyepiece of a stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL) that was focused on the meniscus of the solution in the micropipette. Oxidation potential (+0.55 V for 100 msec) and reduction potential (0 V for 100 msec) were applied alternately to produce a square-wave potential. Electrochemical measurements of

oxidation and reduction currents were made at 5 Hz and averaged to 1 Hz using the Fast16 mkIII system. Stable baseline was defined as 3 consecutive signals for which the maximal amplitudes did not differ by ± 10% following a constant ejection volume of NE solution. For each experiment, 3 to 5 stable signals were recorded.

For experiments in SHR and control strains determining the effect of MPH treatment during adolescence on NET function in adulthood (n = 6–9/group), a single-barrel micropipette containing NE solution (100 μM) with GBR 12909 (50 nM) and ascorbic acid (100 μM) in saline (pH 7.2–7.4) was attached to the Nafion-coated carbon-fiber electrode and lowered into the CG or LO. MPH treatment during adolescence was found previously to increase DAT function in mPFC of adult SHR (Somkuwar et al., 2013). Thus, to ensure that MPH treatment-dependent changes in DAT function did not interfere with MPH-induced alterations in NET function, GBR 12909 was retained in the NE solution in the these experiments. NE clearance was recorded from the four brain regions of interest using a within-subject design. Sequence of recording from mPFC and LO was randomized. Order of recording for CG, PrL and IL subregions of mPFC was sequential as the depth of the electrode placement increased. That is, following 3 to 5 stable signals from one mPFC subregion, the electrode-pipette assembly was moved ventral to the next mPFC subregion.

2.5 Verification of electrode placement

Immediately after each recording session, brains were removed and flash-frozen using 2-methylbutane and stored at -80°C for histological evaluation of microelectrode recording tracks. Brains were sectioned along the coronal plane (20 μm sections) using a Leica 1850 M cryostat (Nussloch, Germany), and sections were thaw mounted onto Fisher SuperFrost Plus® slides. Slides were stored overnight at room temperature under desiccation and stained with cresyl violet for determination of electrode placement. Probe placements (tissue displacement) were evaluated under a light microscope (1 X magnification; Fig 4.1). Position of the tip of the electrode was estimated visually by referencing histological landmarks including corpus collosum, anterior commissure and rhinal fissure while referring to the rat brain atlas (Paxinos, 1991). Only data from histologically confirmed microelectrode placements were included in the subsequent analyses of results.

2.6 Electrochemical data acquisition and statistical analysis

Data were processed using a customized Matlab®-based analysis package, FastAnalysis Version 5.0 (Jason Burmeister Consulting, LLC). The primary parameters for evaluating NE clearance were peak amplitude (A_{max}, in μM) and first-order rate constant (k_{-1}, in sec-1) of the NE signal (Liu et al., 2009). Amount of NE applied was adjusted to ensure that the A_{max} ranged from 0.6 to 1.5 μM and was comparable between subjects. Range of A_{max} was selected apriori based on previous studies evaluating DA clearance by DAT in striatum (Hebert and Gerhardt, 1999) to ensure that extracellular NE concentrations were not saturating. Data for three stable measurements of A_{max} and k_{-1} were averaged for each brain region for each rat. First order uptake rate was calculated as the product of peak amplitude and first order rate constant ($A_{max} X k_{-1}$, in nM/sec; Stephens et al., 2011).

Statistical analyses were conducted using SPSS Statistics Version 19 (SPSS Inc., IBM Company, Armonk, NY). Data are reported as mean ± S.E.M. and n represents the number of rats per group. For the optimization experiments, the dependent variables were compared using paired t-tests. For the MPH treatment experiments, dependent variables were compared using two-factor ANOVA (strain X treatment), followed by Bonferroni's post-hoc comparisons. Outliers were identified using the Grubbs test (GraphPad; http://www.graphpad.com/quickcalcs/Grubbs1.cfm) and were omitted from the analyses.

3 Results

3.1 Histological assessment of electrode placements in mPFC and OFC

Electrode tip placements in mPFC and OFC are illustrated in Fig 1. For LO, electrode placement was between +3.2 to + 4.0 mm anterior and 2.4 to 2.7 mm lateral to bregma. For IL, electrode placement was between +2.7 to +3.2 mm anterior and 0.5 and 1.0 mm lateral to bregma. One WKY rat in the MPH treatment experiment was excluded from the mPFC analyses due to electrode placement outside the acceptable range of co-ordinates.

3.2 NE clearance via NET in the absence and presence of GBR12909 inhibition of DAT

No differences were found in the amount of NE ejected (Supplementary Table S1) or A_{max} (Table 1) when NE was applied in the absence and presence of GBR12909 (50 nM). The average volume of solution that was pressure ejected was 28.6 ± 2.14 nL. For the CG, dorsal PrL, ventral PrL, IL and LO subregions, no differences were found in the first order uptake rate of NE in the absence and presence of GBR12909 (Table 2).

3.3 Effects of methylphenidate treatment during adolescence on NET function in adulthood

No differences were found in the amount of NE applied (Supplementary Table S2) and in the A_{max} (Table 3) between MPH- and VEH-treated SHR, WKY and WIS in CG, dorsal PrL, ventral PrL, IL and LO subregions. The average volume of solution that was pressure ejected into the brain regions was 47.8 ± 4.61 nL.

For first order uptake rate of NE in the LO, a significant strain X treatment interaction was found (F[2,45] = 4.38, p < 0.05; Fig 2). Post-hoc comparisons revealed that the uptake rate in VEH-treated SHR was greater than the uptake rate in VEH-treated WKY and WIS. Importantly, MPH treatment during adolescence reduced the first order uptake rate in adult SHR. No treatment differences were found between the VEH- and MPH-treated WKY and WIS control strains. Further, the uptake rate in MPH-treated SHR was not different from the uptake rate in VEH- and MPH-treated WKY and WIS. In contrast, no differences were found between MPH-and VEH-treated SHR, WKY and WIS in the first order uptake rate of NE in CG, dorsal PrL, ventral PrL and IL (Table 4).

4 Discussion

The current study used an *in vivo* voltammetric method to study NET function in the prefrontal cortex in an animal model of ADHD. The results revealed that NE uptake rate in the LO subregion of OFC was greater in vehicle-treated SHR than in vehicle-treated WKY

and WIS. Importantly, MPH treatment during adolescence followed by cessation of treatment during adulthood decreased the NE uptake rate in LO of SHR, such that uptake rate in MPH-treated SHR was not different from vehicle- and MPH-treated WKY and WIS rats. No effects of strain or treatment were found in subregions of mPFC (including CG, dorsal and ventral PrL, and IL), revealing regional specificity of the effect. The observed increase in NE uptake rate reflects increased NET function, suggesting that adult SHR have reduced extracellular NE and reduced noradrenergic transmission in OFC. Importantly, adolescent treatment with MPH normalized this deficit in noradrenergic transmission detected in adult SHR, and this effect of MPH persisted into adulthood. Thus, MPH treatment during adolescence resulted in long-lasting mitigation of the aberrant noradrenergic transmission in SHR rats with an ADHD phenotype.

The *in vivo* voltammetric method used to evaluate NE clearance within prefrontal cortex was adapted from previously published methods for determining DA clearance within the prefrontal cortex (Zhu et al., 2007). Results revealed that inhibition of DAT function by GBR12909 in mPFC and OFC subregions did not alter first order uptake rate of NE, suggesting that DAT does not contribute significantly to NE clearance in these regions. With respect to the results from the optimization experiments, a large between-subjects variation in uptake rate (Table 2) was observed, despite the within-subject design and the low variation in Amax (Table 1). This was likely due to the small number of subjects and the variation in treatment history of the subjects. Nevertheless, the overall lack of effect of DAT inhibition by GBR12909 on PFC NET function reported herein is consistent with findings from previous report (Moron et al., 2002). Specifically, GBR12909 did not inhibit dopamine uptake into frontal cortical synaptosomes from mice, indicating that NET is responsible predominantly for dopamine uptake in the prefrontal cortex, and the contribution of DAT was minimal (Moron et al., 2002). However, previous studies revealed that MPH treatment during adolescence increased DAT function in the mPFC of adult SHR compared to vehicle control as well as compared to MPH-treated WKY and WIS (Somkuwar et al., 2013). Thus, to avert the possibility that enhanced DAT function in SHR contributes to alterations in NE clearance in prefrontal cortex, the use of GBR12909 was continued in subsequent experiments.

One potential limitation of the measure of first order uptake rate is that it does not take into account the amount of substrate (in this study, NE) applied via micropipette; amount of substrate applied alters A_{max} (Zhu et al., 2007). As a consequence, the amount of NE applied may complicate the interpretation of NET function using uptake rate. Clearance is another measure of transporter function that does account for the amount of substrate applied (Zhu et al., 2013; Zhu et al., 2007). In the current study, there were no significant between-group differences in the amount of NE applied (see Supplementary Table S2), and the range of A_{max} (0.6–1.5 μM) was selected *a priori* based on previous work. Moreover, for studies that control A_{max} between experimental groups, uptake rate has been justified as an indicator of transporter function (Littrell et al., 2012; Miller et al., 2012). Thus, NE uptake rate was considered an appropriate indicator of NET function for the current study.

Increased NET function in the LO subregion of the OFC in the SHR is indicative of a hypofunctional noradrenergic system in this brain region, which may underlie behavioral

deficits observed in the SHR, such as impaired reversal learning, strategy set-shifting and working memory (Harvey et al., 2013; Kantak et al., 2008; Sagvolden, 2006). Further, reduced noradrenergic function in the OFC has been reported to contribute to disruption of goal-directed actions and to increased impulsive behavior (Schwabe et al., 2012; Sun et al., 2010). Reduced noradrenergic function in the OFC may contribute also to ADHD-related behavioral deficits via reduced modulation of noradrenergic and dopaminergic neuronal firing in the locus coeruleus and ventral tegmental area, respectively (Aston-Jones and Cohen, 2005; Chandler et al., 2013). Taken together, the current study contributes to our understanding by identifying an increase in NET function specifically in OFC as a potential neurochemical mechanism for the behavioral deficits in ADHD.

MPH exerts its therapeutic effects, at least in part, by inhibiting NET function and increasing extracellular NE concentrations in prefrontal cortex (Arnsten and Li, 2005; Berridge et al., 2006; Richelson and Pfenning, 1984; Wang et al., 2007). Increased extracellular NE induced by therapeutically relevant doses of MPH activates α2A receptors on cortical pyramidal neurons, and thereby, improves prefrontal cortical-mediated functions including attention and impulsivity (Arnsten and Li, 2005; Bari et al., 2011; Wang et al., 2007). The current results indicate that chronic MPH treatment during adolescence normalizes the elevated NET function in OFC of SHR that persists long after treatment discontinuation.

Of note, MPH treatment during adolescence did not result in persistent alterations in NET function in any prefrontal cortex sub-regions evaluated in control WKY and WIS rats. Previous research has shown that NET expression and function in the forebrain increases from birth to PND 15, but NET expression is steady in adulthood (Coyle and Axelrod, 1971; Sanders et al., 2005). Furthermore, α2-adrenergic receptors in rat cortex follow a similar developmental trajectory (Happe et al., 2004). Thus, the noradrenergic system in prefrontal cortex is developmentally vulnerable during the juvenile period and is relatively stable during adolescence. Therefore, pharmacological manipulation, such as treatment with a low dose of MPH during adolescence, is not expected to produce lasting adaptations to the noradrenergic system in prefrontal cortex in control subjects.

In individuals with ADHD, cortical maturation is delayed during development (Castellanos and Proal, 2012; Posner et al., 2011). As a consequence, the noradrenergic system in the ADHD population may be more susceptible to adaptations following treatment with MPH during adolescence. Furthermore, MPH treatment is often discontinued because several ADHD symptoms diminish with age (Faraone et al., 2006; McCarthy et al., 2009). The current study revealed that MPH treatment during adolescence normalized NET function in an animal model of ADHD. Thus, by extrapolation, MPH treatment in adolescents with ADHD may contribute to the age-related reduction in ADHD symptoms, such that the functional improvement persists long after treatment discontinuation.

The therapeutic benefits of MPH treatment during adolescence may be offset by the long-term consequences on drug abuse risk. Adult SHR exhibit increased cocaine self-administration compared to WKY and WIS (Harvey et al., 2011; Somkuwar et al., 2013). MPH treatment in adolescent SHR followed by treatment discontinuation further increased

cocaine self-administration during adulthood (Harvey et al., 2011) and was associated with increased DAT function in mPFC, but not in OFC (Somkuwar et al., 2013). Similarly, non-medicated adults with ADHD are more vulnerable to drug abuse, including cocaine, compared to individuals without ADHD, which has been attributed to neurobehavioral deficits of ADHD such as enhanced ventral striatal hyporesponsiveness during reward anticipation and enhanced impusivity (Biederman et al., 1998; Lee et al., 2011; Schres et al., 2007). The later in childhood that MPH treatment is initiated, the greater the risk of drug abuse during adulthood, but this association may be moderated by conduct problems in children with ADHD (Dalsgaard et al., 2014; Mannuzza et al., 2008). Consequences of initiation of MPH treatment in a purely adolescent ADHD population have not yet been determined.

5 Conclusion

In conclusion, the current study reveals that NET function is increased in the OFC of SHR and that chronic MPH treatment during adolescence normalized NET function that persisted into adulthood. Elevated NET function in the OFC may contribute to behavioral deficits in SHR. The action of MPH for decreasing NET function may underlie the normalization of ADHD-related behavioral deficits in SHR. Extrapolating these results to individuals with ADHD, increased NET function in the OFC may contribute to the ADHD phenotype, and MPH treatment during adolescence may normalize OFC NET function and result in improvements in ADHD symptoms that may persist long after treatment discontinuation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Methylphenidate (MPH) reduces ADHD by inhibiting norepinephrine transporter (NET)

- Long-term effects of MPH on NET function in orbitofrontal cortex (OFC) are unknown

- NET function was greater in OFC of a rat model of ADHD compared to non-ADHD control

- MPH during adolescence normalized NET function in ADHD model during adulthood

- Thus, some of the therapeutic action of MPH persists long after treatment cessation
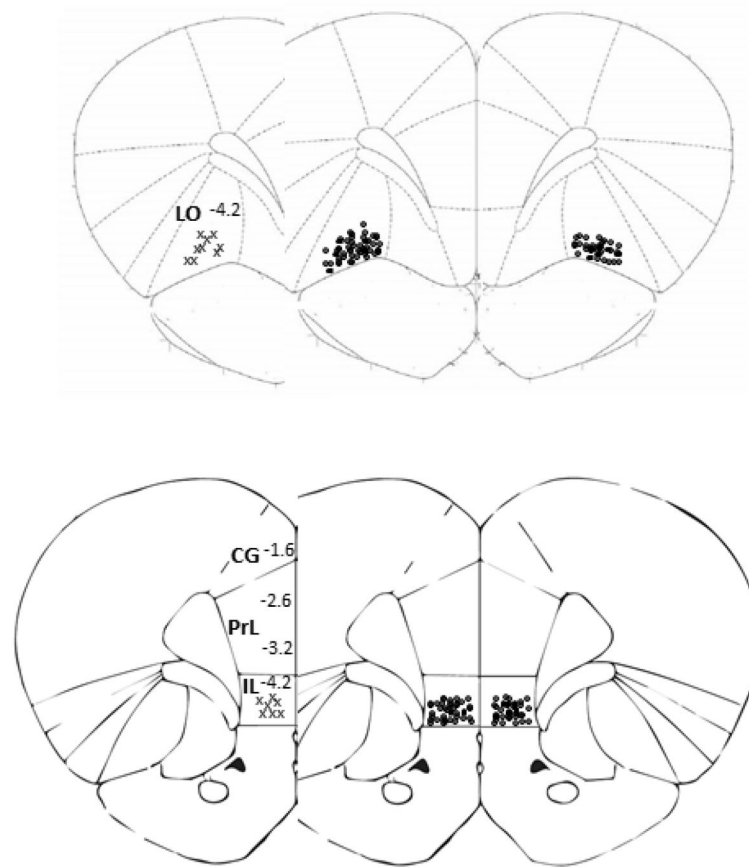
Fig 1.
Neuroanatomical localization of electrodes. The regions of interest for evaluating NET function were lateral orbitofrontal cortex (LO; Top panel), the cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL), and infralimbic cortex (IL; Bottom panel). Placements of the carbon-fiber electrode tips in the rodent LO (Top panel) and IL (Bottom panel) are shown for experiments determining the effect of dopamine transporter (DAT) inhibition (x marks) as well as for the experiments evaluating the effect of adolescent treatment with MPH (black dots).
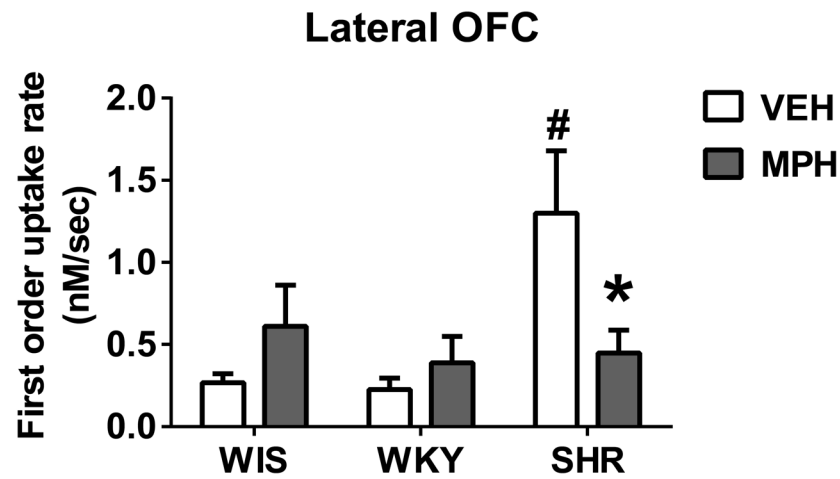
Fig 2.
First order uptake rate of norepinephrine (NE) in the lateral orbitofrontal cortex (LO) of adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats treated with methylphenidate (MPH) or vehicle (VEH) during adolescence. NE (100 μM; with GBR 12909, 50 nM) was applied locally to LO of each rat. First order uptake rate (in nM/sec) was mean $\pm$ S.E.M. product of the maximum amplitude (A_{max}) of the NE signal and the first order fitting of the signal decay (k_{-1}). n = 8–9/group; * $p < 0.05$ compared to the respective VEH control; # $p < 0.05$ compared to VEH-treated WKY and WIS.

Table 1

Maximum amplitude (A_{max}) of the NE signal in the absence and presence of GBR12909 in subregions of mPFC and OFC in Sprague-Dawley rats

Maximum amplitude (A_{max}) of the NE signal in mPFC, including cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL), and infralimbic cortex (IL), and in the lateral orbitofrontal cortex (LO) in the absence and presence of GBR12909. NE (200 μM) was applied alone (NE alone) or with GBR 12909 (50 nM; NE plus GBR12909) in opposite brain hemispheres for each rat. n = 3–4.

	Amax (μM)	
	NE alone	NE + GBR
CG	0.67 ± 0.09[a]	0.79 ± 0.17
Dorsal PrL	1.1 ± 0.04	0.80 ± 0.04
Ventral PrL	1.0 ± 0.25	0.83 ± 0.11
IL	0.80 ± 0.21	0.99 ± 0.10
LO	1.3 ± 0.28	1.7 ± 0.30

[a]Values are mean ± S.E.M.

Table 2

First order uptake rate of NE (A_{max} x k_{-1}) in the absence and presence of GBR12909 in subregions of mPFC and OFC in Sprague-Dawley rats

Uptake rate of NE (200 μM) applied locally to subregions of mPFC, including cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL), and infralimbic cortex (IL), and to the lateral orbitofrontal cortex (LO). NE was applied alone or in the presence of GBR 12909 (50 nM; NE plus GBR12909) in opposite brain hemispheres for each rat. n = 3–4.

	First order uptake rate (nM/sec)	
	NE alone	**NE plus GBR12909**
CG	0.11 ± 0.05^a	0.18 ± 0.10
Dorsal PrL	0.32 ± 0.04	0.21 ± 0.08
Ventral PrL	0.18 ± 0.01	0.20 ± 0.07
IL	0.15 ± 0.09	0.40 ± 0.18
LO	0.72 ± 0.27	0.75 ± 0.20

[a]Values are mean ± S.E.M.

Table 3

Effect of MPH treatment during adolescence on maximum amplitude (A_{max}) of the NE signal in mPFC and OFC of adult SHR, WKY and WIS rats

Maximum amplitude (A_{max}) of the NE signal for subregions of mPFC, including cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL), and infralimbic cortex (IL) and for lateral orbitofrontal cortex (LO) of adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were treated with methylphenidate (MPH) or vehicle (VEH) during adolescence. NE (100 µM, with GBR 12909, 50 nM) was applied locally to each brain region using a within-subject design. n = 7–9/group for CG, dorsal and ventral PrL and LO; n = 6–8/group for IL.

| | A_{max} (µM) | | | | |
	CG	Dorsal PrL	Ventral PrL	IL	LO
SHR-VEH	0.57 ± 0.11 [a]	0.68 ± 0.13	0.69 ± 0.13	0.67 ± 0.09	0.89 ± 0.15
SHR-MPH	0.74 ± 0.13	0.57 ± 0.07	0.68 ± 0.10	0.83 ± 0.17	0.87 ± 0.16
WKY-VEH	1.2 ± 0.33	0.81 ± 0.23	0.97 ± 0.37	0.65 ± 0.12	0.69 ± 0.15
WKY-MPH	0.67 ± 0.12	0.81 ± 1.4	0.86 ± 0.13	0.72 ± 0.11	0.62 ± 0.11
WIS-VEH	1.2 ± 0.28	1.2 ± 0.23	1.3 ± 0.25	1.2 ± 0.38	0.61 ± 0.09
WIS-MPH	1.5 ± 0.52	0.96 ± 0.25	0.73 ± 0.12	0.71 ± 0.07	0.95 ± 0.24
Statistics[b]	$F[2,45]=0.41$	$F[2,44]=0.37$	$F[2,47]=0.31$	$F[2,41]=2.2$	$F[2,45]=0.94$

[a] Values are mean ± S.E.M.;

[b] $p > 0.05$

Table 4

Effect of MPH treatment during adolescence on first order uptake rate of NE in mPFC during adulthood in SHR, WKY and WIS rats

First order uptake rate (A_{max} x k_{-1}) of NE and strain X treatment (3×2) ANOVA for mPFC subregions, including cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL) and infralimbic cortex (IL), of adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were treated with methylphenidate (MPH) or vehicle (VEH) during adolescence. NE (100 μM, with GBR 12909, 50 nM) was applied to each brain region using a within-subject design. n = 7–9/group for CG, dorsal and ventral PrL; n = 6–8/group for IL.

	First order uptake rate (nM/sec)			
	CG	**Dorsal PrL**	**Ventral PrL**	**IL**
SHR-VEH	0.29 ± 0.10	0.43 ± 0.15	0.34 ± 0.10	0.71 ± 0.19
SHR-MPH	0.44 ± 0.15	0.33 ± 0.11	0.48 ± 0.13	0.54 ± 0.13
WKY-VEH	0.50 ± 0.16	0.30 ± 0.06	0.34 ± 0.08	0.34 ± 0.12
WKY-MPH	0.38 ± 0.12	0.35 ± 0.12	0.47 ± 0.11	0.43 ± 0.08
WIS-VEH	0.63 ± 0.15	0.53 ± 0.12	0.56 ± 0.12	0.61 ± 0.19
WIS-MPH	0.71 ± 0.25	0.56 ± 0.30	0.33 ± 0.16	0.19 ± 0.05
Statistics[b]	$F[2,45]=0.39$	$F[2,44]=0.66$	$F[2,47]=1.5$	$F[2,41]=1.5$

[a] Values are mean ± S.E.M.;

[b] $p > 0.05$