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Early postnatal exposure to methylphenidate alters stress reactivity and increases hippocampal ectopic granule cells in adult rats

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Abstract

To mimic clinical treatment with methylphenidate (MPH; Ritalin) for attention deficit/hyperactivity disorder (ADHD), rat pups were injected with MPH (5 mg/kg, I.P.) or placebo twice daily during their nocturnal active phase from postnatal day (PND) 7 to 35. Thirty-nine days after the last MPH administration (PND76), four litters of rats experienced stressful conditions during the 2003 New York City blackout. MPH-treated rats that endured the blackout lost more weight and regained it at a slower pace than controls ($p < 0.05$; $N = 7-11$ /group). Furthermore, MPH-treated rats had elevated systolic arterial blood pressure (from 115.6 ± 1.2 to 126 ± 1.8 mmHg; $p < 0.05$), assessed on PND130 by tail cuff plethysmography. Immunocytochemical studies of transmitter systems in the brain demonstrated rearrangements of catecholamine and neuropeptide Y fibers in select brain regions at PND135, which did not differ between blackout and control groups. However, MPH-treated rats that endured the blackout had more ectopic granule cells in the hilus of the dorsal hippocampal dentate gyrus compared to controls at PND 135 ($p < 0.05$; $N = 6$ /group). These findings indicate that early postnatal exposure to high therapeutic doses of MPH can have long lasting effects on the plasticity of select brain regions and can induce changes in the reactivity to stress that persist into adulthood.

Keywords

Ritalin; ADHD; blood pressure; hippocampus; ectopic granule cells; stress; blackout

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*Conflict of Interest

The authors declare that they have no competing financial interests

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1. INTRODUCTION

Millions of school children are prescribed methylphenidate [(MPH); Ritalin] to alleviate symptoms of attention deficit/hyperactivity disorder (ADHD), including hyperactivity and reduced attentiveness [1;31]. Although the symptomatic benefits of MPH in ADHD are well documented, little is known about the long-term effects of MPH on brain and behavior. Recently, we published the short- and long-term effects of this drug on brain transmitter systems and behavior in rats [postnatal day (PND) 7 – 35] to model therapeutic doses of MPH given to children with ADHD [13]. We found that immediately after administration of MPH (PND 35) neurotransmitter systems located in brain areas involved in motivated behaviors, cognition, appetite and stress were changed. In particular, more catecholamine fibers and Nissl stained cells and fewer norepinephrine transporter (NET) containing fibers were found in the deep layers of the medial prefrontal cortex (mPFC) of MPH-treated rats. In hippocampal dentate gyrus, rats receiving MPH showed a similar decrease in NET-immunoreactivity as well as an expanded distribution of the new born cell marker [6] polysialic acid-neural cell adhesion molecule (PSA-NCAM). These observations suggest that MPH delayed maturation in these regions (see discussion [13]). In support of this, a recent study reported that brain cortical maturation, as measured by thickness, is attained at a later age in children diagnosed with ADHD [43]. Interestingly, in this study 66% of the patients were previously treated with psychostimulants such as MPH.

In other brain areas, we previously reported that on PND35 MPH-exposed rats also had decreased catecholamine fiber density in the medial striatum and increased neuropeptide Y (NPY) fiber density in hypothalamus [13]. When assessed three months after discontinuation of MPH (PND135), most of the anatomical changes were not apparent but MPH-exposed rats exhibited decreased anxiety in the elevated plus-maze and a trend for decreased catecholamine-containing fibers in the mPFC. Thus, these studies demonstrated that although the observed neuroanatomical changes following early post-natal MPH administration largely resolved with time, some behavioral effects persisted into adulthood.

In the course of performing the above study, four litters of rats treated with MPH and corresponding controls experienced an unplanned stressor: the 2003 New York City blackout, which happened about 6 weeks after discontinuing MPH (PND 76). This short-lasting stressful event had protracted effects on selected metabolic, functional and anatomical systems reported herein. Some of the data presented here have been previously published in an abstract [14].

2. MATERIALS AND METHODS

2.1 Animals

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee. Timed-pregnant Sprague Dawley female rats (N = 8) were obtained from Taconic Farms (Chatham, NY, USA). Mother rats were housed individually in free-standing “Shoe-box” cages (model #6, dimensions: 30.80 cm × 59.37 cm × 22.86 cm; Thoren Caging Systems Inc., Hazleton, PA) and kept at an ambient temperature of 70 – 75°F in a reverse light cycle room (lights on 11:00 pm and lights off 11:00 am) equipped with red (GBX-2) darkroom safelights (Eastman Kodak, Rochester, NY). Day of birth was considered PND 0. After weaning at PND 21 until the experiment ended, MPH and control rats were housed in the same room. Specifically, littermate male rats (N = 40) were pair-housed in cages stacked in the same ventilator rack (cage model #4, dimensions: 30.80 cm × 30.80 cm × 18.72 cm; Thoren Caging Systems Inc.). During normal operation, the air exchange rate inside the cages was 80 changes/hour. All animals had free access to food pellets

(kept in the tops of the cages) and water (which was in water bottles throughout the study.) A total of 4 litters were exposed to the New York City Black out.

2.2 MPH Administration

The details for the administration procedures and choice of MPH model have been described in detail previously [13]. Briefly, the litters were sexed and culled to 10 pups per litter on PND 3. Males and female pups were included until weaning to maintain a consistent litter size and normal male-female composition. To distinguish control and MPH-administered rats, the paws of each pup were tattooed with India ink [5]. From PND 7 – 35, litter-mate sex-matched pairs of rat pups were weighed and injected with either 5 mg/kg d, l-threo-MPH (obtained from NIDA Research Triangle Institute, Research Triangle Park, NC) in saline or an equal volume of saline I.P. twice daily, six hours apart during their dark (e.g., active) cycle. At the end of the administration phase (PND35), male littermate pairs of rats from each group were weighed every 3 or 4 days until the conclusion of the experiment (PND135). Repeated measures of weight were evaluated for systematic variation by litter, using repeated measures analysis of variance (RMANOVA). Differences were considered significant when $p < 0.05$.

2.3 Blood pressure measurements

Systolic arterial blood pressure (ABP) measurements from awake MPH injected and control rats were made using tail-cuff plethysmography [19] by a person blind to the experimental conditions. On PND130, a total of 10 ABP measurements were recorded over a one-hour session using a non-invasive tail cuff sphygmomanometer (Kent Scientific Corporation, Torrington, CT). The values were averaged to determine the final systolic ABP for the day. Since the experimental design entailed relative comparisons within groups of rats, the fact that tail-cuff plethysmography can underestimate absolute ABP values [15] did not compromise the results. Data were expressed as averages $\pm$ standard error of the mean and all four groups were analyzed using a nested univariate ANOVA with litter as a random factor in the model followed by Tukey post hoc analysis. Differences were considered significant when $p < 0.05$. Accurate blood pressure measurements were obtained from a total of 10 MPH treated animals and 7 control animals.

2.4 Immunocytochemical procedure

Section Preparation—On PND135, littermate pairs of male rats, each pair consisting of a MPH-administered and a saline-administered rat, were deeply anesthetized with sodium pentobarbital (150 mg/kg) and their brains fixed by aortic arch perfusion sequentially with: a) 10 – 15 ml saline (0.9%) containing 1000 units of heparin; b) 50 ml of 3.75% acrolein and 2% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.6); and c) 200 ml of 2% paraformaldehyde. Following the perfusion, the brains were removed and cut into 5 mm coronal blocks using a brain mold (Activational Systems, Inc., Warren, MI), and post-fixed for 30 min in the latter fixative. The brains were sectioned on a Leica VT1000X Vibratome (40 μ m thick) and stored in cryoprotectant (30% sucrose, 30% ethylene glycol in PB) at -30° C until processing. Prior to immunocytochemical procedures, sections from littermate rat pairs were rinsed in PB, coded with hole-punches and pooled into single containers. Rostrocaudal levels of the paired sections were matched so that near-identical brain regions could be compared between groups. Sections were treated with 1% sodium borohydride in PB for 30 min to neutralize free aldehydes and rinsed thoroughly in PB. Sections then were rinsed in 0.1M Tris-buffered saline (TS; pH 7.6) and incubated for 30 min in 0.5% bovine serum albumin (BSA) in TS. For immunocytochemical procedures, 3 pairs (1 control and 1 MPH animals) from litter 1, 1 pair from litter 2 and 2 pairs from litter 4 were included in the analysis for a total of 6 animals per treatment group. All animals included in the immunocytochemical procedure had accurate measurements of blood pressure.

Brain regions analyzed and corresponding atlas levels—Similar to our previous study [13] sections containing brain regions implicated in ADHD, hyperactivity and/or learning and attentional processes were selected for analysis using the Swanson atlas [44] as a guide. Fourteen regions were examined: amygdala (central and basolateral nuclei; levels 27 – 28; AP = –2.00 to –2.45 from bregma); anterior cingulate cortex (levels 22 – 24; –4.60 to –1.33 from bregma); globus pallidus (levels 20 – 22; –0.46 to –4.60 from bregma); hippocampal dentate gyrus (levels 28 – 38; –2.45 to –5.65 from bregma); dorsomedial (DMH) and paraventricular (PVN) hypothalamic nuclei (levels 23 – 30; –1.08 to –3.25 from bregma); locus coeruleus (levels 51 – 52; –9.80 to –10.10 from bregma); nucleus basalis (levels 17 – 23; –0.0 to –1.08 from bregma); prefrontal cortex (dorsal and medial; levels 8 – 10; +3.20 to +2.15 from bregma); raphe nuclei (dorsal; levels 44 – 45; –7.60 to –7.90 from bregma); septal nuclei (medial septum/diagonal band of Broca; levels 15 – 16; +0.45 to +0.10 from bregma); striatum (13 – 16; +1.20 to +0.10 from bregma); substantia nigra and ventral tegmental area (levels 37 – 39; –525 to –6.06 from bregma).

Antisera—Antisera and cytological stains were selected to identify neurotransmitter systems implicated in ADHD and/or targeted by MPH and to identify particular cell populations that develop postnatally are important for development. Details for antisera characterization and specificity are provided in the indicated references. Monoaminergic neurons and processes were identified using a mouse monoclonal antibody to the catecholamine synthesizing enzyme, tyrosine hydroxylase (TH; diluted 1:10,000), obtained from Incstar [22]. Dopaminergic neurons and processes were identified using a polyclonal rat antiserum to the dopamine transporter (DAT; diluted 1:5000), obtained from Chemicon [42]. Noradrenergic neurons and processes were identified with a rabbit polyclonal antiserum to the NET (diluted 1:20,000), supplied by Dr. Randy Blakely [24]. Serotonin neurons and processes were identified with a goat polyclonal antiserum to the serotonin transporter (SERT; diluted 1:5,000), obtained from Santa Cruz [7]. Cholinergic neurons were identified by using a goat polyclonal antibody to vesicular acetylcholine transporter (VACHT; diluted 1:20,000), obtained from Diasorin [3]. Select neuropeptides were labeled using (1) a monoclonal mouse antibody to Leucine-enkephalin (L-Enk; diluted 1:3,000) obtained from Diasorin [10]; and (2) a polyclonal rabbit antiserum to NPY (diluted 1:2000) obtained from Peninsula [23]. Cell-specific markers were used in this study. Nissl substance in cell bodies was stained using Thionin. Fibrous astrocytes were identified using a mouse monoclonal antibody to glial fibrillary acidic protein (GFAP; diluted 1:6000) obtained from Chemicon [6]. Hippocampal granule cell nuclei were labeled using a rabbit polyclonal antibody to the divergent member of the basic-helix-loop-helix family of transcription factors-homeobox protein PROX1 (diluted 1:10,000) obtained from Chemicon [11]. A goat polyclonal antibody to doublecortin (DCX; diluted 1:2000) obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and a mouse monoclonal antibody to PSA-NCAM (diluted 1:500) obtained from Chemicon were employed to label the carbonate portion of the highly polysialylated neural cell adhesion molecule on newly born cells in the dentate gyrus [6;12].

Immunocytochemical processing—Coronal sections containing the desired brain areas from pairs of experimental rats were processed for the immunocytochemical localization of the above antisera using the avidin-biotin complex (ABC) method [16]. In brief, tissue sections were incubated for 1 day at room temperature and for 1 day at 4°C in the predetermined dilution of antiserum (see above) in 0.1% BSA in Tris-saline (TS; pH 7.6). All sections except those incubated in PSA-NCAM were incubated in a 1:400 dilution of biotinylated anti-immunoglobulin (IgG) against the species in which the primary antiserum was raised (Jackson ImmunoResearch Inc., West Grove, Pennsylvania) BSA/TS for 30 minutes. Sections labeled for PSA-NCAM were incubated in biotinylated anti-mouse IgM (Cymbus Biotech., Flanders, NJ). Following incubation in the secondary antibody, the sections were rinsed in TS and

incubated in ABC solution (at twice the recommended dilution; Vector Laboratories; Burlingame, CA) for 30 min followed by development in 3,3'-diaminobenzidine (Aldrich Chemical Co., Milwaukee, Wisconsin) and H₂O₂ in TBS for 6 minutes. Sections were mounted on gelatin-coated slides, dried in a desiccator. The slides then were dehydrated through a graded ethanol series and coverslipped from xylene with DPX neutral mountant for histology (Aldrich Chemical Co.).

Data analysis—Initially sections were analyzed qualitatively on a Nikon Eclipse 80i light microscope equipped with bright-field and differential interface contrast optics and a Micropublisher digital camera (Q Imaging, Barnaby, British Columbia). For the qualitative analysis, low magnification (2X or 4X) photomicrographs were taken of the brain region of interest (ROI) in each littermate pair of sections. Photographs were laid side-by-side to determine whether MPH administration possibly altered the pattern or density of a particular marker in the ROI. If differences were seen in the pattern of labeled fibers, higher magnification (20X) photographs were taken of the ROI and quantitative densitometry was performed on the ROI using Image-J using previously described methods [26]. Final densities were obtained by subtracting the average pixel density for 2 regions that lacked labeling from the average pixel density of 2 ROI measurements. Data were expressed as averages $\pm$ standard error of the mean and first analyzed for litter effects using a nested univariate ANOVA. For comparisons between two treatment groups, if no effect of litter was observed, an independent sample Student t-test was used.

For cell counts (e.g., PROX, PSA-NCAM, DCX) in the hippocampal dentate hilus, sections were coded and analyzed by an investigator unaware of the experimental groups. Low magnification (4x) photographs were taken of the mid-septotemporal level (approximately –3.9 from Bregma) of the dentate gyrus and all cells (or nuclei for PROX) between the two blades of the granule cell layer were counted. Data was analyzed as mentioned above.

For final photomicrographs, digital images were adjusted for brightness and contrast in Adobe Photoshop 7.0 on a Macintosh G5 computer. Photographs were taken with the same light intensity and adjustments were made so that background intensities were identical. Graphs were prepared with Graph Pad Prism 4.01 (Graph Pad Software, Inc., San Diego CA); quantitative data are represented as average $\pm$ SEM. Final figures were generated using Quark X-Press 6.1. Statistical analyses were performed using SPSS for Windows V.11.0.1 (SPSS Inc., Chicago, IL).

3. RESULTS

3.1 During the New York City blackout the housing cage environment was altered

During the New York City blackout the microenvironment of the cages was altered for approximately 18 hours. After the start of the blackout (approximately 4:00 pm EST 8/14/03), all animals experienced slightly higher ambient temperatures (from 75°C at 11:00am on 8/14/03 to 79°C at 11:00am on 8/15/03), failure of the day/night cycle reversal equipment (one light cycle was reduced from 12 hours to 1 hour) and of the oxygen exchange system. However, soon after the onset of the blackout, the cages were opened to prevent hypoxic conditions (e.g., build-up of CO₂ levels). The humidity level (67%) inside the room housing the cages was in the range observed the week prior to the New York City blackout (65 – 70%).

3.2 Early post-natal MPH administration influenced body weight responses following the New York City blackout

In the present cohort of rats, the New York City blackout occurred on PND76, about six weeks after discontinuing MPH administration. As described in a previous report [13], the weight

gain showed significant interaction with litter, with differential rates of weight gain across litters. Weights from 7 times of measurement (Days 65, 71, 74, 78, 80, 86, 88) were submitted to a repeated measures analysis of variance (RM-ANOVA) with time (weight) as the within-subjects (repeated) factor, group as the between-subjects factor, and litter as a covariate. We predicted that the time-by-group interaction would be significant, as that is relevant to the question of differential response to the blackout experience. Our predictions held: main effect of time and the time-by-group interaction effect achieved significance ($F(6, 14) = 3.28$; $p < 0.05$; Fig. 1). Following the blackout, MPH-administered rats lost weight, while control animals continued to gain weight. Specifically, MPH-treated animals showed a 14% decrease in weight while control rats showed a 24% increase in weight relative to 9 days previous to the blackout. For this same time-period, we previously showed that the rate and magnitude of weight gain was not significantly different between control and MPH-administered rats that did not experience the New York City blackout [13]. To further clarify the change in weight directly associated with the blackout *per se*, 6 change scores (Day 65 to 71, 71 to 74, etc.) were calculated using multiple regression. Residualized scores were calculated by regressing weight from Day $t+1$ on weight from Day t , using the approach described by Lewis and Ramsay (1995) [20]. Residualized scores were standardized with $M = 50$ and $sd = 10$ and were submitted to one-way ANOVAs with treatment group as the independent variable. Significance level was defined using the Bonferroni adjustment. Alpha was defined as $p = .0083$ ($.05/6$). Only 1 of the 6 change scores achieved significance: the change in weight from Day 74 to Day 79 or pre- to post-blackout ($F(6, 14) = 3.28$, $p = .008$). In essence, the weight gain in animals from the MPH group deviated significantly from a linear trend, while the weight gain in animals from the control group maintained a linear trend.

3.3 Developmental MPH administration differentially influenced ABP measurements in adult rats that endured the New York City blackout

Three months after the MPH administration period (PND 130), ABP was measured using tail-cuff plethysmography from rats that had endured the New York City blackout and from rats maintained in normal housing conditions. The latter group of rats was the same cohort of animals examined at PND135 in our previous study [13]. No effect of litter was observed ($F(12, 18) = 0.58$, $p > 0.5$). ANOVA revealed a main effect of group ($F(3, 17) = 4.86$, $p < 0.05$; Fig. 2). Post hoc analysis revealed that MPH-administered rats that had endured the New York City blackout had significantly increased ABP when compared to controls that also endured the blackout ($p < 0.05$). For both groups of rats that were maintained under normal conditions, no differences in ABP were observed ($p > 0.05$). Although qualitative observations suggest that rats in the control group that were maintained under normal conditions had higher ABP than the control group that endured the New York City blackout, these groups were not different from each other ($p = 0.342$).

3.4 No differences in neurotransmitter systems and brain cytoarchitecture were observed

Consistent with previous observations in rats housed under normal conditions [13], no statistical differences ($P > 0.5$) were observed in either the levels or distributions of catecholaminergic- (e.g., TH, NET, DAT and SERT) or cholinergic- (e.g., VACHT) fibers in the prefrontal cortex, hippocampal dentate gyrus, and caudate and in neuropeptide Y-containing fibers in the hypothalamus 3 months after MPH-administration (Table 1; Fig. 3). Moreover, no significant differences ($P > 0.05$) in the number of doublecortin and PSA-NCAM (markers of new cells) and in the density of GFAP (astrocytic marker) were found between MPH-administered and control rats in the dorsal hippocampus (Table 1).

3.5 More ectopic hippocampal granule cells were found in MPH rats that had endured the New York City blackout

To identify granule cell bodies the distribution of PROX-labeled nuclei was examined. All PROX-immunoreactive nuclei lying $> 15 \mu\text{m}$ (about the width of one large nucleus) outside the granule cell layer on the hilar side of the dentate were counted as “ectopic”. In agreement with earlier studies [11], PROX-immunoreactive nuclei were most prominent in the granule cell layer of all rats, regardless of experimental condition (Fig. 4 A, B). However, in contrast to our previous studies which examined rats under normal housing conditions [13], significantly more PROX-labeled nuclei were found in the hilus ($t(10) = 2.32$, $p < 0.05$), particularly the subgranular zone, of MPH-administered rats that had undergone the blackout compared to their littermate controls (Fig. 4B, C).

4 DISCUSSION

This study reveals that early postnatal exposure to high therapeutic doses of MPH can enhance metabolic, functional and anatomical responses to stress. In particular, six weeks after the discontinuation of MPH administration, rats that received MPH lost more weight, regained it more slowly, had elevated ABP and had increased numbers of hippocampal ectopic cells as compared to controls who were exposed to the same stressors during the New York City blackout.

4.1 Developmental MPH affects weight and ABP responses following New York City blackout stress

MPH administration during pre- and peri-adolescence can alter behavioral responses to stressors in humans [4]. We showed previously that administration of MPH to rats resulted in decreased time spent in the open arms of the radial arm maze as compared to control animals when tested three months after MPH administration had ceased, suggesting that the MPH-treated rats were less anxious [13]. Besides behavioral measurements, little evidence is found in the literature regarding the effects of stressor weight gain or arterial blood pressure once MPH treatment has stopped. One previous study has shown that tail pinch stress in rats reverses the anorexia induced by MPH acutely; no long term effects were documented [2]. However, in children, it is well documented that MPH can cause anorexia and delayed growth (for review see [32]). Both animal and clinical studies have shown that the growth suppressing effects of MPH are acute and reversible after drug administration has stopped [29;30;33]. Even after individuals “catch up” on growth after discontinuation of MPH, our results on weight gain or arterial blood pressure point towards long-term effects on their reactivity to stressors.

Several clinical reports have shown that continuous administration of MPH elevates blood pressure in children [34;46] and heart rate and blood pressure in adults [47]. Some of these initial changes in blood pressure can dissipate with time [35] or the effects are minimal and non-significant as compared to control animals [13]. However, to our knowledge, long-term effects of MPH treatment on heart rate or arterial blood pressure have not been reported. The present study shows for the first time that the effects on arterial blood pressure are persistent for at least 6 weeks after MPH administration ceased in animals that were stressed during the New York City blackout. One study raises the possibility that early stress and MPH can cross-sensitize dopaminergic responses showing that early exposure to stressful life events can modulate MPH-induced dopamine release in the medial prefrontal cortex and nucleus accumbens [18]. Jezierzky and colleagues also suggest that the opposite phenomenon is true: early exposure to MPH can modulate stress-induced dopamine release [18]. There is also evidence that dopaminergic activity can modulate the levels of angiotensin present in the brain [21] which may affect blood pressure regulation in response to stress in MPH pretreated animals. While the mechanism of how MPH treatment during early development affects ABP

in response to stress is not completely clear, we postulate that it is likely to involve sensitization of dopamine release which has been shown previously to be induced by pharmacological treatments with psychostimulants [28]. However, it is also possible that the observed MPH effects on blood pressure occur through peripheral mechanisms.

4.2 More ectopic granule cells are found in the dentate gyrus of developmentally administered MPH rats exposed to the New York City blackout

The present study revealed that in rats that endured the blackout, MPH-administered rats had more ectopic granule cells as compared to controls. The majority of these ectopic granule cells were found in the dentate gyrus subgranular zone. Interestingly, we [13] previously observed that immediately following one month of MPH administration this same region had a significant wider distribution of immunoreactivity for PSA-NCAM, a marker of new cells [6]. Since granule cells develop from these PSA-NCAM containing progenitor cells [41], our findings suggest that MPH administration to developing rats may position some progenitors farther away from their intended destination of the granule cell layer rendering them more likely to become ectopic. Although our previous study found no difference in the number of ectopic granule cells in control and early postnatal administered MPH rats assessed as adults three months after discontinuing the drug [13], the added stressor of the blackout may have additionally affected the proliferation and/or migratory paths of new cells in the dentate gyrus. This in turn could account for the higher incidence of ectopic granule cells observed in the MPH administered animals in the present study. In support of this conclusion, many forms of stress can affect cell proliferation, survival and new neuron formation in the hippocampal formation of many species (for review see [25]). Moreover, even an acute episode of naturally occurring stress can reduce the survival of newly generated neurons [45].

Ectopic granule cells have most frequently been reported after seizures (for review see [37]). Ectopic granule cells in the subgranular zone from both seizure and normal animals have been shown to extend into and branch within the molecular layer [9;39]. Although these integrate into the dentate circuitry [17] and have physiological properties similar to granule cells [39], there are some differences. In particular, ectopic granule cells are contacted by a high number of excitatory-type mossy fiber terminals and discharge spontaneous bursts of action potentials, features thought to predispose them to discharge in epileptiform bursts [27]. Thus, although the function of ectopic granule cells seen in the MPH administered rats that underwent the New York City blackout is not completely clear, it is possible that they could affect the excitability and general function of the hippocampal formation.

4.3 Other considerations

The conditions following the New York City blackout were not under our control and produced significant changes in environment, such as altered light/dark cycle and increased CO₂ in the housing environment. The environmental stressors produced were mild and cannot be directly compared to other more common laboratory controlled stressors such as electric footshock, restraint stress or water baths. However, a single change in light/dark cycle has been shown to increase brain-derived neurotrophic factor (BDNF) in the rat hippocampus [40]. BDNF, which is prominent in the hilus [38], has been shown to increase neurogenesis and ectopic granule cells [36]. Thus, it is interesting to speculate that changes in BDNF levels may have contributed to our observed increases in ectopic cells following the blackout.

4.4 Conclusion

Our findings are consistent with the views that MPH targets brain regions and functions beyond those implicated in executive function in ADHD pathology [8]. This study also adds to the growing body of evidence showing that MPH exposure during early development affects reactivity to stressors and challenging situations later in life.

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Fig. 1. Following the New York City blackout body weight was more affected in rats that had received early postnatal MPH

Six weeks after cessation of treatment, male rats that received MPH and experienced the New York City blackout lost weight instead of continuing to gaining weight as compared to control rats that also endured the New York City blackout. Inset shows changes in the weight gain at PND 79 relative to days preceding the black out (PND 74–78). $p < 0.05$; $N = 7-11$ per group; bars represent average and SEM

Fig. 2. Three months after cessation of administration, rats that received MPH and also endured the New York City blackout had significantly higher ABP

Three months after cessation of treatment (approximately PND130), MPH administration during development (PND7 - 35) significantly influenced ABP in male rats that had endured the New York City blackout. * = $p < 0.05$ (post hoc); bars represent averages and SEM. Numbers inside bars represent animals/group.

Fig. 3. Distribution of catecholamine fibers or neuropeptide Y was not different 3 months later (PND135) after MPH administration in animals that endured the New York City blackout
A. Density of TH-immunoreactive fibers in the medial prefrontal cortex (PFC) of MPH-administered rats (A₂) compared to controls (A₁). Atlas level 9, AP = +2.80 from Bregma. cc: corpus callosum. **B.** TH-immunoreactivity in coronal sections of striatum (atlas level 16, AP = +0.10 from Bregma) in control (B₁) and MPH administered rats (B₂). **C.** Controls (C₁) and MPH-administered rats (C₂) had similar densities of TH-labeled fibers in the central hilus (ch). Gcl: granule cell layer, sgz: subgranular zone. Atlas level 32, AP = -3.90 from Bregma. **D.** NPY-immunoreactive fibers in the PVN in MPH-treated rats (D₂) compared to controls (D₁).

Atlas levels 26, AP = -1.78 from Bregma. All atlas references from [44]. Pictures were adjusted for brightness and contrast. Bar A and C: 50 μ m; B: 200 μ m; D: 250 μ m

Fig. 4. More ectopic granule cells were observed in adult rats that had received MPH during development and had endured the New York City blackout

Photomicrographs of the dorsal hippocampal dentate gyrus (atlas level 32, AP = -3.90 from Bregma [44]) labeled with PROX in a control (A) and MPH-administered rat (B) that had endured the New York City blackout. In both conditions, PROX-labeled nuclei were most prominent in the granule cell layer (GCL). However, more PROX-labeled nuclei (arrows) were found in the hilus (hil), especially the subgranular zone of the MPH-administered rats. Bar, 100 μ m. C. A significant increase (*) in the number of PROX-labeled nuclei in the hilus was seen in MPH administered rats compared to controls. * = $p < 0.05$ (independent sample t-test), N = 6/condition. Pictures were adjusted for brightness and contrast. Bar, 50 μ m.

Table 1

Changes in neurotransmitter systems and brain cytoarchitecture produced by developmental exposure to MPH as compared to control group. All animals experienced NYCBO environmental stressor.

Label	Region assessed	MPH rats
Monoamines		
tyrosine hydroxylase	PFC, ACC, PVN, DMH, CP, GP, DG, AMY, SN/VTA	no differences
dopamine transporter	CP, GP, SN, VTA	no differences
norepinephrine transporter	PFC, DG, PVN, DMH, LC	no differences
serotonin transporter	PFC, ACC, DG, DR	no differences
Acetylcholine		
vesicular acetylcholine transporter	PFC, ACC, MS/DB, NB, DG, AMY	no differences
Neuropeptides		
neuropeptide Y	PVN, DMH, DG	no differences
Cell markers		
Thionin	PFC, ACC, LV, CP, DG	enlarged LV (N = 1)
PROX	DG	increased nuclei in hilus
PSA-NCAM/DCX	DG	no differences
GFAP	DG	no differences

Abbreviations: ACC, anterior cingulate cortex; AMY, amygdala; CP, caudate putamen; DG, hippocampal dentate gyrus; DMH, dorsomedial nucleus of the hypothalamus; DR, dorsal raphe; GP, globus pallidus; LC, locus ceruleus; LV, lateral ventricle; MS/DB, medial septum/diagonal band; NB, nucleus basalis; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus; SN, substantia nigra; VTA, ventral tegmental nucleus.