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Chronic oral methylphenidate treatment increases microglial activation in rats

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Abstract

Methylphenidate (MP) is a widely prescribed psychostimulant used to treat attention deficit hyperactivity disorder. Previously, we established a drinking paradigm to deliver MP to rats at doses that result in pharmacokinetic profiles similar to treated patients. In the present study, adolescent male rats were assigned to one of three groups: control (water), low-dose MP (LD; 4/10 mg/kg), and high dose MP (HD; 30/60 mg/kg). Following 3 months of treatment, half of the rats in each group were euthanized, and the remaining rats received only water throughout a 1-month-long abstinence phase. In vitro autoradiography using [³H] PK 11195 was performed to measure microglial activation. HD MP rats showed increased [³H] PK 11195 binding compared to control rats in several cerebral cortical areas: primary somatosensory cortex including jaw (68.6%), upper lip (80.1%), barrel field (88.9%), and trunk (78%) regions, forelimb sensorimotor area (87.3%), secondary somatosensory cortex (72.5%), motor cortices 1 (73.2%) and 2 (69.3%), insular cortex (59.9%); as well as subcortical regions including the thalamus (62.9%), globus pallidus (79.4%) and substantia nigra (22.7%). Additionally, HD MP rats showed greater binding compared to LD MP rats in the hippocampus (60.6%), thalamus (59.6%), substantia nigra (38.5%), and motor 2

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Conflict of interest The authors declare that they have no conflict of interest.

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cortex (55.3%). Following abstinence, HD MP rats showed no significant differences compared to water controls; however, LD MP rats showed increased binding in pre-limbic cortex (78.1%) and ventromedial caudate putamen (113.8%). These findings indicate that chronic MP results in widespread microglial activation immediately after treatment and following the cessation of treatment in some brain regions.

Keywords

Methylphenidate; Ritalin; Attention deficit hyperactivity disorder; Autoradiography; Microglia; Inflammation

Introduction

Methylphenidate (MP) is one of the most frequently prescribed psychostimulant medications for treating attention deficit hyperactivity disorder (ADHD) (Greenhill et al. 2002a, b). ADHD is a neurodevelopmental disorder affecting approximately 11% of school aged children, representing a 41% increased prevalence rate over the past decade (Visser et al. 2014). Additionally, studies on illicit use of MP show that up to 30% of college students surveyed report using MP for cognitive enhancement, weight loss, or its euphoric effects (Bogle and Smith 2009; Lakhan and Kirchgessner 2012; Fallah et al. 2018). The increasing use and abuse of MP causes concern over its long-term effects, especially since MP is most often used during critical periods of neurodevelopment. Previously, we found that chronic MP, provided at doses that mimic the pharmacokinetic profile of treated patients, results in a myriad of effects on physiology (including skeletal development) (Komatsu et al. 2012; Uddin et al. 2018; Robison et al. 2017b; Thanos et al. 2015), behavior (Robison et al. 2017b; Thanos et al. 2015), and neurochemistry (Robison et al. 2017a) in normal rats, with some effects persisting beyond the end of treatment. There is a concerning gap in knowledge, however, as to whether chronic MP treatment could result in significant brain inflammation, as has been shown following chronic administration of related psychostimulants (Cadet et al. 2005; Thanos et al. 2016a).

MP's mechanism of action is partially mediated by its ability to block dopamine (DA) and norepinephrine (NE) transporters and increase extracellular DA and NE (Zhang et al. 2012; Volkow et al. 2002). Clinical results have shown increases in extracellular DA in the prefrontal cortex and striatum following MP administration (Zhang et al. 2012; Volkow et al. 2001). In rats, chronic MP has been shown to alter binding levels of the dopamine transporter and receptors in several regions of the basal ganglia, while a prolonged abstinence period reverses these effects (Caprioli et al. 2015; Robison et al. 2017a). Additionally, it has been shown that MP treatment results in changes in catecholamine systems within the medial prefrontal cortex, hippocampus, striatum, and hypothalamus (Gray et al. 2007). Due to an increase in cytokines and chemokines, excess dopamine has been shown to induce an inflammatory response in the brain, which could lead to an induction of microgliosis (Jang et al. 2012). Therefore, one possible consequence of MP treatment that has not been fully explored is its ability to trigger inflammatory and neurodegenerative processes, as has been shown to result from chronic administration of

related psychostimulants (Gonçalves et al. 2010; Thanos et al. 2016a; Cubells et al. 1994; Cadet et al. 2005).

Studies have confirmed that MP reduces the expression of neurotrophins (Sadasivan et al. 2012), and induces apoptosis, oxidative damage, and DNA fragmentation in brain cells (Banihabib et al. 2014; Andreazza et al. 2007; Motaghinejad et al. 2016, 2017a, b; Gomes et al. 2008). Acute and chronic MP also increases the expression of proinflammatory cytokines, while chronic MP increases microglial activation in several brain regions including the cortex, hippocampus, and basal ganglia (Sadasivan et al. 2012; Motaghinejad et al. 2016, 2017b). Chronic administration of clinically relevant doses of MP change cell density and morphology in the dentate gyrus and CA1 of the hippocampus (Motaghinejad et al. 2016) and causes a 20% reduction in dopamine neurons in the substantia nigra pars compacta (SNpc) (Sadasivan et al. 2012). Additionally, MP treatment sensitizes SNpc neurons to the parkinsonian agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as measured by increased cell death, and enhanced the number of activated microglia following lesioning (Sadasivan et al. 2012). Taken together, the aforementioned results suggest that MP treatment could result in neuroinflammation and neurodegeneration across several brain regions; however, there are a few methodological concerns of previous studies. First, all of these studies administered MP intraperitoneally, which differs significantly from oral administration, specifically with respect to time to peak serum concentration, half-life, and rate of elimination (Kuczenski and Segal 2001), as well as absolute magnitude and time course of increases in extracellular DA (Kuczenski and Segal 2001; Gerasimov et al. 2000). Additionally, it is of interest to determine whether the detrimental effects of MP persist beyond the cessation of treatment, as many children taking MP discontinue treatment in later adulthood.

The current study employs a previously established oral dosing paradigm to deliver MP to adolescent rats at two clinically relevant doses (4/10 mg/kg and 30/60 mg/kg) to reproduce the pharmacokinetic profile observed in treated humans (Thanos et al. 2015). The goal of this study is to assess the effects of chronic MP (3 months of treatment) on neuroinflammation in rats, as measured by microglial activation throughout the whole brain, as well as the possible impact of cessation of treatment (following a 1 month drug abstinence period).

Methods and materials

Animals

Adolescent 2-week-old male Sprague Dawley rats (Taconic, Hudson, New York, USA) were individually housed in a controlled room (22 ± 2 °C and 40–60% humidity) with a 12-h reverse light–dark cycle (lights off 0800 h). All rats were allowed 1 week of habituation before starting on the MP drinking paradigm. Food was provided ad libitum throughout the entire experiment. All works were conducted in conformity with the National Academy of Sciences Guide for Care and Use of Laboratory Animals (National Academy of Sciences NRC, 1996) and approved by the University at Buffalo Institutional Animal Care and Use Committee.

Drugs

Methylphenidate hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in distilled water to produce several doses (as previously described and shown below) of 4, 10, 30, and 60 mg/kg solutions. Individual rats' drinking bottles were made fresh daily based on body weight and the average of their last 3 days' fluid intake to achieve desired dosages.

Drug paradigm

Rats were split into three groups, receiving water (control), low dose (LD, 4 mg followed by 10 mg) MP, or high dose (HD 30 mg followed by 60 mg) MP treatment ($n = 15$ – 16 for all groups). During the treatment period, rats had access to fluids during their dark cycle for 8 h per day. During the first hour of access (09:00 h–10:00 h), 4 mg/kg MP (LD MP) or 30 mg/kg (HD MP) drinking solutions were provided, following by drinking solutions at concentrations of 10 mg/kg (LD MP) or 60 mg/kg (HD MP) for the remaining 7 h (10:00 h–17:00 h). This treatment was based on a previously established dual-bottle 8-h limited access drinking paradigm, which results in a pharmacokinetic profile similar to that observed in patients treated with MP (peaking at ~ 8 – 10 ng/mL for the LD MP group and ~ 25 ng/mL for the HD MP group) along with similar liquid intakes among the treatment groups (Thanos et al. 2015). Following a 3-month treatment period, half of the rats in each group were euthanized and their brains were extracted for analysis. The remaining half of the animals went through a 1 month abstinence period, during which all rats received only water for the 8-h limited access drinking period while continuing to have access to food ad libitum.

Procedures

[^3H] PK 11195 autoradiography

24 h following the respective treatment or abstinence period, rats were anesthetized using isoflurane ($\sim 3.0\%$). Brains were quickly removed, flash-frozen in 2-methylbutane, and stored at -80°C until use ($n = 7$ – 8 /group). Cryostat sections ($14\ \mu\text{m}$ thick) were cut, mounted on slides, and stored tightly sealed at -80°C in the presence of desiccant, until the day of the binding experiment. [^3H]PK 11195 binding was carried out according to a previously established protocol (Thanos et al. 2016b). Sections were pre-incubated for 15 min in 50 mM Tris–HCl buffer (pH 7.4) at room temperature. Sections were then incubated in 50mM Tris–HCl buffer (pH 7.4) with the addition of 0.8nM [^3H] PK 11195 (85.7 Ci/mmol, PerkinElmer Inc.) for 30 min at room temperature. Non-specific binding was determined on consecutive sections in the presence of excess 20 μM unlabeled PK 11195. After incubation, sections were washed twice for 6 min in ice-cold 50 mM Tris HCl buffer (pH 7.4) and then dipped in ice-cold distilled water.

After binding, all sections were dried under a stream of cool air and exposed onto Kodak BioMax MR Film for 4 weeks, alongside calibrated tritium standards (American Radiolabeled Chemicals, St. Louis, MO). Following exposure, the films were developed in Kodak D-19 developer, dried and scanned as TIFF images at 1200 dpi, under uniform conditions. All regions of interest were quantified using the calibrated standard curves with Image J software (NIH). Regions of interest (ROI) selected for analysis include major areas of the cerebral cortex: prelimbic/infralimbic, cingulate, retrosplenial, insular, piriform,

ectorrhinal, perirhinal, lateral entorhinal, motor (M1 and M2), primary (jaw, upper lip, barrel field, trunk) and secondary somatosensory (S2), sensorimotor (hindlimb, forelimb), auditory, and visual. Subcortical areas selected for analysis include: nucleus accumbens, amygdala, striatum [split into dorsolateral (DL), dorsomedial (DM), ventrolateral (VL), and ventromedial (VM) quadrants], globus pallidus, hippocampus, hypothalamus, thalamus, and substantia nigra. See Fig. 1 for a map of ROIs drawn.

Statistics

Specific binding for each ROI was analyzed with one-way ANOVA, followed by Tukey's post hoc comparisons when appropriate. All statistical tests were run using SigmaPlot 11.0 software (Systat software Inc., San Jose, CA), with statistical significance set at $\alpha = 0.05$.

Results

[3H] PK 11195 binding following 3 months of MP treatment

[3H] PK 11195 specific binding following 3 months of MP treatment was analyzed for each ROI with one-way ANOVA, with treatment as factor (Water, LD, HD) (Figs. 2, 3, 4, 5). A significant effect of treatment was observed for the insular cortex [$F(2,20) = 4.488$; $p < 0.05$]; motor 1 cortex (M1) [$F(2,20) = 5.899$; $p < 0.05$]; motor 2 cortex (M2) [$F(2,20) = 5.565$; $p < 0.05$]; jaw somatosensory cortex [$F(2,20) = 5.068$; $p < 0.05$]; forelimb sensorimotor cortex [$F(2,20) = 5.218$; $p < 0.05$]; upper lip somatosensory cortex [$F(2,20) = 5.435$; $p < 0.05$]; whisker somatosensory cortex (barrel field) [$F(2,20) = 5.032$; $p < 0.05$]; trunk somatosensory cortex [$F(2,19) = 3.974$; $p < 0.05$]; secondary somatosensory cortex (S2) [$F(2,20) = 4.347$; $p < 0.05$]; globus pallidus [$F(2,19) = 4.004$; $p < 0.05$]; hippocampus [$F(2,20) = 4.445$; $p < 0.05$]; thalamus [$F(2,20) = 5.643$; $p < 0.05$], and substantia nigra [$F(2,20) = 5.298$; $p < 0.05$].

Tukey's post hoc tests found that HD MP treated rats showed significantly greater [^3H] PK 11195 binding compared to water treated rats in the insular cortex, M1, M2, forelimb sensorimotor, jaw, upper lip, barrel field and trunk S1 areas, S2, globus pallidus, thalamus, and substantia nigra ($p < 0.05$ for all). Additionally, HD MP treated rats showed increased [^3H] PK 11195 binding compared to LD treated rats in the M2, hippocampus, thalamus, and substantia nigra ($p < 0.05$ for all).

[3H] PK 11195 binding following 1 month abstinence from MP

[3H] PK 11195 specific binding following 3 months of MP treatment and 1 month abstinence was analyzed for each ROI with one-way ANOVA across treatment (Water, LD, HD) (Figs. 2, 3, 4, 5). A significant effect of treatment was observed in the prelimbic cortex [$F(2,18) = 4.044$; $p < 0.05$] and ventromedial striatum [$F(2,18) = 3.975$; $p < 0.05$]. Tukey's post-hoc test found that LD treated rats showed increased [^3H] PK 11195 binding compared to water treated rats in these two regions ($p < 0.05$ for both).

Discussion

Here, we show that chronic daily treatment with a clinically comparable high dose of MP for 3 months can cause increases in microglial activation in multiple regions throughout the brain, (including the primary and secondary somatosensory cortices, primary and secondary motor cortices, insular cortex, hippocampus, thalamus, globus pallidus, and substantia nigra); however, PK 11195 binding levels in HD MP rats were indistinguishable from control rats following a 1 month abstinence period. In contrast, the LD MP treated rats showed increased PK 11195 binding and microglial activation compared to control rats in prelimbic cortex and ventromedial striatum following the abstinence period. These results suggest that chronic MP treatment could result in neuroinflammation of specific brain regions.

An increase in microglial activation in the somatosensory cortex, motor cortices, basal ganglia, and thalamus could have downstream effects on sensorimotor function. It has been shown that MP is capable of modulating responses of the somatosensory cortex as well as regional blood flow (Drouin et al. 2006, 2007; Lee et al. 2005). Additionally, chronic MP modulates locomotor activity and sensory evoked responses in freely behaving rats (Yang et al. 2006). The insular cortex is involved in a variety of functions including pain perception, speech production, and the processing of social emotions (Nieuwenhuys 2012). The effects of chronic MP on social behavior have also been explored, with variable results in that some found no effect (Robison et al. 2017b), while several others suggest a disruption in social behavior (Vanderschuren et al. 2008; Trezza et al. 2009; Thor and Holloway 1983; Achterberg et al. 2015). Finally, HD MP treated rats displayed increased binding in comparison to LD MP treated rats in the hippocampus, a region associated with short-term declarative memory and spatial learning and memory (Purves and Fitzpatrick 2001), suggesting that chronic high dose MP use could result in cognitive dysfunction. Previous studies have shown that chronic MP treatment impairs performance on memory tasks such as the Morris water maze (Scherer et al. 2010), object place (LeBlanc-Duchin and Taukulis 2009), and novel object recognition (Scherer et al. 2010; Heyser et al. 2004; LeBlanc-Duchin and Taukulis 2009); however, others report no effects (Bethancourt et al. 2009; Robison et al. 2017b) or even improvements (Bethancourt et al. 2009) in hippocampal-dependent memory, likely due to differences in subjects and/or dosing regimens (routes of administration).

Following an extended 1-month abstinence period, these effects were reversed and there were no longer any differences in [^3H] PK 11195 binding between HD MP and water treated rats. Interestingly though, the chronic LD MP treated rats, even after a 1 month abstinence period, showed significant increased microglial activation in the pre-limbic cortex (78.1%), a medial prefrontal area implicated in attention and cognitive flexibility (Dalley et al. 2004), and the ventromedial striatum (113.8%), which is critical for reward-guided and habitual behavior.

[^3H] PK 11195 is a peripheral benzodiazepine receptor antagonist (Venneti et al. 2006) and binding has been reported to correspond to activated microglia in brain tissue following various neuronal injuries in rodent models (Stephenson et al. 1995; Pedersen et al.

2006;Raghavendra Rao et al. 2000; Vowinckel et al. 1997). Microglia will become activated in response to CNS damage that stimulates these cells to function as phagocytes (Gehrmann et al. 1995). Activated microglia can be cytotoxic producing potentially cytotoxic molecules such as nitric oxide and proinflammatory cytokines (Nakajima and Kohsaka 2001). In addition to their phagocytic role, microglia also have a protective role for the brain promoting neurogenesis (Butovsky et al. 2006) and removing toxic glutamate levels (Persson et al. 2005). The present findings can be compared to studies on the neurochemical effects of other psychostimulants such as cocaine and methamphetamine which both increase extracellular dopamine levels (Volkow et al. 1997; Sulzer et al. 2005). Excess dopamine could be toxic, producing both post and pre-synaptic damage to structures within the brain (Filloux and Townsend 1993). An increase in cytokines and chemokines characterize an inflammatory response in the brain, which has been seen following an increase in free dopamine (Jang et al. 2012), and which could lead to an induction of microgliosis. Stimulant drugs such as methamphetamine have been proven to induce microglial activation after treatment (Thomas et al. 2004; Thanos et al. 2016a), as well as increase other markers of inflammation and neurodegeneration processes (Gonçalves et al. 2010;Thanos et al. 2016a;Cubells et al. 1994; Cadet et al. 2005), while chronic MP treatment in normal animals has reported to lead to structural changes such as decreasing the volume of posterior white matter tracts (Delis et al. 2017).

Some aspects of the study, aside from MP treatment, may have influenced the results of the binding assay. Rats in this study were single housed to accurately measure fluid and food consumption throughout the entirety of the experiment. Single housing is understood to be a “deprived” environment, which may influence the results since animals in socially isolated environments have been shown to be more sensitive to psychostimulants (Howes et al. 2000).

Increasing rates of non-prescription MP use among healthy individuals have been reported (Swanson and Volkow 2008), especially for academic improvement and euphoric effects (Lakhan and Kirchgessner 2012) and it has been reported for over a decade that stimulant use is widespread in college students (Arria and DuPont 2010). Therefore, it is important to further study the neurodevelopmental effects of chronic MP administration. Moreover, among college students nonmedical use of prescription stimulants was associated with past year illicit drug use, alcohol dependence, and marijuana dependence (Arria et al. 2008). Finally, illegal or prescription use of MP is also sought as a mean of decreasing appetite leading to weight loss based on limited findings in the pediatric population with ADHD (Schwartz et al. 2014). The present study found that chronic MP treatment increased microglial activation in multiple brain regions in rats, including the cerebral cortex, hippocampus, thalamus, and basal ganglia. While many of these effects were reversible by long-term abstinence, some inflammation appeared (lose dose MP) beyond the cessation of treatment. These findings present grave concerns over the use and abuse of MP due to its ability to trigger inflammatory and possibly neurogenerative processes, particularly during critical periods of childhood through young adulthood while the brain is still developing.

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Fig. 1. Autoradiographic coronal brain images of [3H] PK 11195 binding following 3 months of treatment between water (control), low dose methylphenidate (MP), and high dose MP at the following bregma coordinates: + 4.20, + 1.08, – 2.04, and– 5.16 mm, along with the corresponding regions of interest examined drawn on reference brain atlas images as adapted from Paxinos and Watson rat brain atlas

Fig. 2.

Mean [3H] PK 11195 specific binding in somatosensory cortex of control (water), low dose (LD) methylphenidate (MP), and high dose (HD) MP treatment groups following 3 months of treatment (a) or a 1 month abstinence period following 3 months of MP treatment (b). *J* Jaw, *FL* forelimb, *ULp* upper lip, *HL* hindlimb, *BF* barrel field, *TR* trunk region, *S2* secondary somatosensory. [^] $p < 0.05$ compared to water group

Fig. 3.

Mean [3H] PK 11195 specific binding in cortical regions of control (water), low dose (LD) methylphenidate (MP), and high dose (HD) MP treatment groups following 3 months of treatment (a) or a 1 month abstinence period following 3 months of MP treatment (b). *IL* Infralimbic, *PrL* prelimbic, *Cg* cingulate, *Ins* insular, *M1* primary motor, *M2* secondary motor, *Aud* auditory, *Vis* visual, *Rs* retrosplenial. [^] $p < 0.05$ compared to water group; [#] $p < 0.05$ compared to LD group

Fig. 4. Mean [3H] PK 11195 specific binding in subregions of the basal ganglia in control (water), low dose (LD) methylphenidate (MP), and high dose (HD) MP treatment groups following 3 months of treatment (**a**) or a 1 month abstinence period following 3 months of MP treatment (**b**). *DM* Dorsomedial, *DL* dorsolateral, *VM* ventromedial, *VL* ventrolateral, *Nac* nucleus accumbens, *GP* globus pallidus, *SNR* substantia nigra. [^] $p < 0.05$ compared to water group; # $p < 0.05$ compared to LD group

Fig. 5.

Mean [3H] PK 11195 binding in control (water), low dose (LD) methylphenidate (MP), and high dose (HD) MP treatment groups following 3 months of treatment (a) or a 1 month abstinence period following 3 months of MP treatment (b). *Piri* Piriform cortex, *Ect* ectorrhinal cortex, *PRh* perirhinal cortex, *L Ent* lateral entorhinal cortex, *Amyg* amygdala, *Hippo* hippocampus, *Thal* thalamus, *Hypo* hypothalamus. [^] $p < 0.05$ compared to water group; [#] $p < 0.05$ compared to LD group