Effects of methylphenidate (Ritalin) on anxiety, locomotor activity, and cognition in pre- and peri-adolescent rats

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Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
DA	Dopamine
DAT	Dopamine Transporter
EA	Enclosed Arm
i.p.	Intraperitoneal
MPD	Methylphenidate
NE	Norepinephrine
NET	Norepinephrine Transporter
OA	Open Arm
PFC	Prefrontal Cortex
PND	Postnatal Day
PSAP	Protected Stretch-Attend Posture
RAM	Radial Arm Maze

Abstract

Attention deficit/hyperactivity disorder (ADHD) is a disorder affecting approximately 9% of school-aged children. A number of therapeutic options exist to treat ADHD. One, methylphenidate (MPD), improves behavior of patients with ADHD in real life, as well as their performance in laboratory settings. Recent evidence indicates that MPD affects healthy humans in a manner similar to that in ADHD patients. However, data regarding the mechanisms and effects of MPD are inconsistent, particularly with respect to age groups (i.e., children, adolescents, adults). Using the rat as a model, we conducted several studies to clarify the effects of oral MPD on cognitive function, activity and anxiety during pre- and peri-adolescence in animals.

A preliminary study revealed apparent age-dependent and multifaceted effects of oral MPD in pre- and peri-adolescent rats. There, we found that daily oral administration of MPD at 3 mg/kg resulted in: **(1)** improved performance from postnatal day (PND) 22 to 24 but impaired performance from PND 32-34; **(2)** increased locomotor activity and reduced anxiety-like behaviors at PND 22-23; and **(3)** improved performance in an attentional task. Moreover, we found that responses to oral MPD at 3 mg/kg varied among rats, specifically in locomotor activity and performance in the attention task.

Here, we examined the following three hypotheses stemming from these preliminary data: **(1)** the effects of oral MPD at clinically-relevant doses on performance in cognitive tasks are age-dependent; **(2)** oral MPD alters anxiety-related behaviors and locomotor activity in an age-dependent manner, both of which contribute to the effects of MPD on performance in cognitive tasks; **(3)** individual responses to novelty (i.e., new environments) in rats predict individual behavioral responses to oral MPD, including cognitive performance, anxiety-like behaviors, and locomotor activity.

To test our hypotheses, we tested drug-naive pre- and peri-adolescent rats over three age ranges (PND 23 to 28, PND 29 to 34 and PND 36 to 41) for **(1)** the effects of oral MPD in a radial arm maze

(RAM) task; **(2)** the effects of oral MPD on anxiety-related behaviors and locomotor activity on the elevated plus-maze; and **(3)** interaction effects between treatment conditions and individual responses to novelty on measures from the behavioral tests. Plasma and brain levels of MPD were also measured to examine the absorption and metabolism of the drug across these age ranges.

We found that **(1)** oral MPD at 3 mg/kg improved RAM performance during PND 36-41 but not during PND 23-28 and PND 29-34; **(2)** oral MPD at 3 mg/kg reduced anxiety-related behaviors across all ages and increased locomotor activity only on PND 24 and 37; and **(3)** individual response to novelty was only correlated with overall locomotor activity on the elevated plus-maze, not with anxiety-related behaviors or cognitive performance. Brain and plasma levels of MPD were highest on PND 24, lower on PND 30, and lowest on PND 37. In addition, the higher dose of oral MPD improved RAM performance significantly during PND 36-37.

These results suggest that, during pre- and peri-adolescence: **(1)** oral MPD at a clinically-relevant dose produces multifaceted effects that change across ages, which together contribute to the improved performance on cognitive tasks; **(2)** the effects of oral MPD vary according to the state of maturation of the individual; and **(3)** these effects, according to known brain circuit information, are likely through independent circuits. Therefore, under the conditions of our testing paradigm, the radial arm maze, the effects of MPD on anxiety and locomotor activity are more substantial than the effects on cognition.

Introduction

Attention deficit/hyperactivity disorder (ADHD) is a prevalent disorder that impairs a broad set of domains of function and reduces patients' quality of life. The disorder affects 5 to 10% of the population [3-5] and about 9% of school-aged children, according to a recent study based on parent-report rating [6]. Academic and social difficulties emerge in children with ADHD when they reach school age and continue into adolescence [7]. Adulthood ADHD has only recently been recognized [8].

The Questions about ADHD

A number of unresolved questions abound in the field of ADHD. Most concern the mechanisms behind ADHD pathology: What is the underlying cause(s) of the disorder? What neurotransmitter(s) is the key player(s), and how does it behave (e.g., hyper- or hypo-functional) in ADHD? What is the biomarker(s) of ADHD (e.g., gene or pathological features)? Is it possible to make a more objective diagnosis? What mechanism(s) underlies the therapeutic actions of the available medications?

Of all these questions, the grand quest in the field of ADHD is to identify a core functional deficit and the underlying structural change that explains the heterogeneous behavioral symptoms of the disorder. A unifying theory of the disorder is necessary for facilitating objective clinical practice, as well as for advancing research on the etiology, biomarkers, and therapeutic interventions of ADHD. However, it is, in fact, this heterogeneity of ADHD on behavioral and structural levels that is driving the field from seeking a uni-core theory to considering multipathway models.

Heterogeneous Behavioral Symptoms of ADHD

Currently, ADHD remains a behaviorally-defined disorder for which a diagnosis is made based on onset, duration, and level of severity of behavioral symptoms [9]. The defining symptoms of ADHD fall into two categories, namely hyperactive and/or impulsive and inattentive behaviors [10]. ADHD is subdivided into three types: predominantly hyperactive/impulsive, predominantly inattentive, and the combined type [9]. Fifty to seventy-five percent of individuals with ADHD fall in the combined subtype, 20-30% fall in the inattentive subtype, and less than 15% fall in the hyperactive-impulsive subtype [8;11-13].

The hyperactive/impulsive dimension involves a higher level of movements of body parts and the whole body compared to typical individuals [14;15]. However, the expression of hyperactivity might depend on the context [16]. Overactivity compared to healthy controls is seen in structured school activities such as the classroom or during performance of laboratory-based attention tasks [16-18], but not when allowed to play [16] or in novel situations [19;20]. Since displaying inappropriate (level of) activity in a given context is conceptualized as impulsivity, a school of researchers argue that hyperactivity in ADHD is a behavioral expression of impulsivity.

The attention problems of children with ADHD are often described as "distractibility" and trouble with "sustaining attention". However, the behavioral problem may be rooted in more profound dysfunction [21]. Laboratory testing detects various deficits in ADHD patients that are not limited to attention. The most replicated results include deficits in response inhibition, processing speed, working memory, set shifting, planning, and organization [for review see 22].

Recently, an emotional dimension has been argued as a core symptom of ADHD. Since the 1970s, ADHD has been associated with emotional problems that do not meet clinical diagnostic criteria [23]. Children and adults with ADHD, as reviewed by Sobanski et al. [24], display "irritability, hot temper, low frustration tolerance, and sudden unpredictable shifts towards negative emotions". Additionally, co-morbidities such as anxiety and mood disorders occur in about 25% and 15-75% of patients, respectively [25;26].

Heterogeneous Neurocognitive Deficits of ADHD

Laboratory testing demonstrates deficits in individuals with ADHD that fit into four themes. First, executive function, is a set of cognitive skills that can be further separated into inhibitory control, planning, set-shifting, and working memory [27;28]. Second, motivation can be defined as “a process that influences the direction, persistence, and vigor of goal-oriented behaviors” [29]. Third, temporal information processing is “the ability to order sequential events in time and the ability to create rhythms; skills that depend on intact time perception, time discrimination and time (re)production” [30]. Finally, emotional processing may be defined as the ability to register the significance of an event with magnitude and urgency [31].

Children with ADHD reportedly show deficits in all domains of executive function [22;32-34]. Additionally, research suggests that children with ADHD have low levels of intrinsic motivation [35] and/or altered sensitivity to reward/reinforcement (extrinsic motivation) [36;37], e.g., ADHD children often prefer immediate, small reward over large, delayed reward [38]. Children with ADHD display abnormal temporal processing [39;40], which may cause the frequently seen high intra-individual variability of reaction time [41-43]. Further, both children and adults with ADHD have deficits in processing emotional stimuli, such as perceiving emotional facial expressions and contextual description [44-48].

Several early hypotheses of ADHD inferred that executive or motivation/reward dysfunction gives rise to deficits in the other three domains, as well as the behavioral symptoms of ADHD. Barkley [32] proposed that deficits in behavioral inhibition, or inhibitory control, were the core of ADHD, giving rise to four secondary deficits in behavior including “(a) working memory,(b) self-regulation of affect-motivation-arousal, (c) internalization of speech, and (d) reconstitution”. The motivation/reward-centered views of ADHD hypothesized that impulsivity, hyperactivity, and inattention are behavioral expressions of the patients' aversion of delayed reward/outcome [27;37;49]. These theories helped the transition toward more modern theory-driven research for ADHD.

However, other human studies indicated that the four domains of deficits may be independent. Sonuga-Barke [40;50] showed that in children and adolescents with ADHD, inhibitory control, temporal processing, and/or delay-related deficits co-occurred at chance level. Others [51;52] showed that executive deficits did not correlate with delay-related deficits. Abnormal emotional processing was found after controlling for deficits in executive function [44;47;48], but more studies are needed on the relationship of emotional processing to the other three domains of function.

The separability of deficits in executive function, motivation/reward, temporal processing, and possibly emotional processing, indicates that ADHD may result from disruption of multiple, relatively-independent pathways. When one (or more) pathway is disturbed severely enough, the patient displays clinically-significant symptoms of ADHD. The most promising candidate pathways include those in charge of motor control, executive function, motivation/reward, temporal perception, and emotional processes.

Multipathway Neuroanatomy of ADHD

Investigations into the mechanisms behind ADHD have uncovered several neuroanatomical differences in affected individuals. Structural and functional magnetic resonance imaging (MRI) has repeatedly revealed abnormalities in at least three structures in ADHD patients, specifically the prefrontal cortex (PFC), striatum (caudate and putamen, and nucleus accumbens located ventrally), and the cerebellum [reviewed in 53]. Abnormalities of amygdala, hippocampus, and thalamus have been reported but far less frequently [reviewed in 54]. This heterogeneity in observed defects again points to a multipathway mechanism of ADHD.

The PFC, striatum, and cerebellum regulate those domains of function most affected in ADHD. The PFC is central to structures mediating executive functions of response inhibition, working memory, and set-shift [55;56]. Further, the PFC reciprocally connects with other structures, such as amygdala, hippocampus, and thalamus, which contribute to loops in learning and memory, reward, and emotional processes [57-61]. Together with the striatum, the PFC regulates motivational/reward processes, goal-directed movements, and interference control (inhibitory control and orientation of attention) [62-64]. Additionally, the PFC, striatum, and cerebellum are all indicated in motor-timing processes [for a review, see 65;66;67;68;69].

Although these structures are consistently identified as affected, the changes reported are contradictory. Compared to controls, a majority of studies showed that patients with ADHD have decreased volumes [70-73] and hypoactivation [74-76] of PFC, caudate, putamen, nucleus accumbens, and the cerebellum (vermis) [reviewed in 53;77;78;79;80;81]. A few studies, however, showed larger than normal size and hyperactivation of these structures [74;82;83]. Deviant intra- and inter-regional connectivity is also reported among the PFC, striatum, and the cerebellum compared to controls [84;85].

Meanwhile, very few imaging studies have addressed neuroanatomical changes accompanying emotional processes in ADHD. Nonetheless, these studies reported alterations of multiple structures. A consistent finding in ADHD, although from the same group, is that negative emotional stimuli (pictures of faces or words) induce activation of the dorsolateral PFC and deactivation of the ventralmedial PFC [86;87]. Children and adults with ADHD showed smaller regional grey matter volumes [88;89], changes in morphology [90], or no change in the amygdala and hippocampus. fMRI study found a deactivation of the insula and no abnormal amygdala activation [91] upon viewing negative pictures (e.g., wounded children, scenes of violence) compared to neutral ones. Another study reported increased amygdala activity to neutral faces compared to the controls when children with ADHD were rating subjective fear of facial expressions [92]. More studies are needed to determine the roles of the PFC and limbic structures in emotional dysregulation in ADHD, such as whether abnormal activation of PFC leads to abnormal reaction to emotional stimuli or abnormal reaction from limbic structures lead to the deviant activation of the PFC.

Overall, ADHD is associated with a delay in brain development. The total cerebral and cerebellar volumes determined by structural MRI are smaller in people with ADHD compared to controls, including both grey and white matter [71;93]. Further, cortical thickness of those with ADHD reaches peak value later in the middle PFC (5 years) and the superior and medial PFC (2 years) than typically-developing controls [93]. However, Shaw et al. [94] found that developmental trajectories of cortical thickness did not differ significantly between ADHD and control groups. This suggests an early-onset developmental delay rather than a deviance [95].

A Developmental, Systemic, and Dimensional View of ADHD

At first glance, the separability of domains of behavioral deficits seems contradictory to the relatively consistent data of alterations in the PFC, striatum, and cerebellum, and the overall cortical developmental delay. However, as true for most studies analyzing multiple parameters with variability, large changes in merely a portion of subjects can yield statistically significant results for the whole group. In Sonuga-Barke’s studies finding separable behavioral deficits [40;50], group analysis of subjects still yield significant impairment in inhibitory control, motivation, timing, and working memory.

Similarly, separable neuroanatomical alternations may also exist underneath the consistent pattern derived from the population of ADHD.

Taking behavioral and anatomical data together, ADHD may represent an extreme of a continuum of disturbed brain development affecting mostly the fronto-striatal and/or fronto-cerebellar networks. That is, a considerable number of people may have components of ADHD-like deficits and developmental imbalance in the brain to some extent, but only those who have chronically-impaired brain development would develop significant structural abnormalities and meet diagnostic criteria for ADHD.

Consistent with the developmental characteristics of ADHD, a genome-wide association meta-analysis study revealed that “45 of the 85 top-ranked ADHD candidate genes encode proteins” involved in outgrowth of dendrites and axons [96]. The authors of the meta-analysis also pointed out that several of these proteins, e.g., AVP [97] and CREB5 , are reportedly modulated by methylphenidate, a commonly used medication for ADHD.

My hypothesis is that ADHD begins with an early developmental delay. Following the developmental delay, one or more of the aforementioned three structures may be affected more than the rest of the brain, perhaps due to a genetic predisposition (e.g., [98]). The differentially affected regions would produce an imbalance of function in the whole brain. The out-of-balance structure(s) may further affect other reciprocally-connected structures. Abnormal development then reaches a point where the top-down control (e.g., from PFC) and/or other systems can no longer compensate for the impaired domain(s) of function. Behaviorally, such an individual would display chronic deficits that are inappropriate for his or her age.

Accordingly, the structure(s) that is developmentally "singled-out" determines which set of deficits an individual will display. For example, a PFC-centered developmental disturbance could lead to poor executive functions (poor behavioral planning and inhibitory control) and top-down attention control deficits (failure to orient responses), which would put the individual in the predominantly inattentive subtype. In contrast, a striatum-centered developmental disturbance could cause impaired modulation of goal-directed movements, reinforcement learning and memory, which may put the individual in the predominantly hyperactive/impulsive subtype. Similarly, a cerebellum-centered developmental disturbance could cause impaired modulation of motor functions, learning, and timing, which may put the individual in the predominantly hyperactive/impulsive subtype as well.

Impairments in the aforementioned domains of function could give rise to apparent hyperactivity, impulsiveness, and inattentiveness in behavior. As mentioned, the PFC reciprocally connects to subcortical structures such as amygdala, hippocampus, and thalamus, which also participate in learning and memory, reward, and emotional processes [58]. Compensating for one or more domains of function may undermine the PFC (and related structures) in its own function and development, thereby giving rise to multiple sites of abnormality in imaging and combined types of deficits in behavior, even co-morbidities. A simplified relationship between affected structures, domains of function, and downstream behavioral expression are illustrated in **Fig. 1**.

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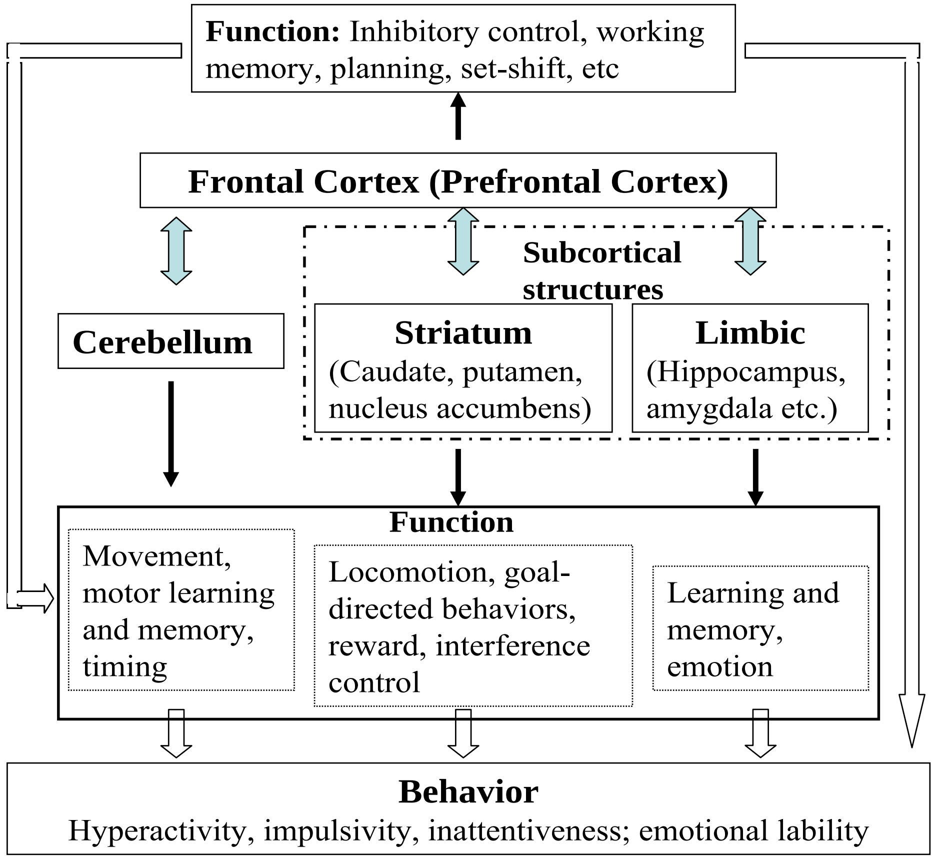

Figure 1. A simplified diagram of the relationship among the structures and function domains involved in ADHD. The PFC, striatum, cerebellum, and limbic system form parallel loops that mediate motor, cognitive, and affective behaviors. These structures interact with each other through direct and indirect reciprocal pathways, which form a dynamic network. The PFC has broad, primarily inhibitory influence over the other structures. A disturbance severe enough in the PFC, striatum, and/or cerebellum can lead to dysfunction spreading to the in-network structures. Lasting severe dysfunction produces an ADHD diagnosis.

In further analysis of the genetic component, a number of researchers, led by Levy in 1997 [99], have proposed a continuum view of ADHD. In a large-scale genetic twin study, Levy concluded that "ADHD is best viewed as the extreme of a behavior that varies genetically throughout the entire population rather than as a disorder with discrete determinants." Such a developmental and multi-system hypothesis of ADHD suggests that multiple factors, from genetic to environmental, can influence the net outcome of the developing brain. Indeed, several studies have associated various factors with ADHD, including variants of a number of genes coding components of dopamine (DA), norepinephrine (NE) [100;101], and serotonin systems [102], environmental toxins such as maternal smoking and pesticide, socioeconomic status, and parenting. Most studies on risk factors of ADHD found significant but small associations of factors with ADHD diagnosis. These data suggest that each individual factor significantly contributes to the manifestation of ADHD but cannot singly account for it. This notion is in line with a multi-contributor neurobiology of ADHD within which individual factors have additive effects.

Interestingly, all of these data regarding the structural, genetic, and environmental contributors to ADHD pathology indicate that a drug for treating ADHD may have to act through multiple pathways to improve behavioral symptoms of the heterogeneous population of ADHD. Methylphenidate (MPD), one of the most prescribed drugs for ADHD, may be of one of the drugs that act through a multi-site/pathway mechanism.

The Multifaceted Effects of Methylphenidate in ADHD

MPD is a widely-prescribed psychostimulant for treatment of ADHD [4;103]. Approximately 2.8% of children and adolescents aged 5 to 18 in the United States have a prescription for Ritalin [104;105] , the best-known form of MPD in North America. Often, patients take MPD for years [106;107].

Literature reporting the effects of MPD on ADHD dates back to the 1970s. Long-term clinical treatment with MPD reduces symptoms of hyperactivity, impulsiveness, and inattention and improves social performance of affected children [108-110]. Indeed, the National Institute of Mental Health (NIMH) Multimodal Treatment (MTA) study of children with ADHD trial on MPD revealed that 14 months of treatment with MPD was significantly superior to behavioral therapy for reducing parent and teacher rating of ADHD symptoms [111].

Data on the effects of MPD on emotional function is relatively limited. Existing clinical studies generally show a positive effect of long-term MPD in adult ADHD but a more ambiguous effect in pediatric ADHD. Adult ADHD studies revealed that MPD is superior to placebo in reducing emotional lability [112-117]. In pediatric ADHD, several studies reported that short (>1 week) to long-term (<24 weeks) MPD treatment is associated with reduction of feeling worried, anxious, irritable, and "crabby", but also with an increase in sadness, depression, and social withdrawal [118-126]. The MTA study indicated an effect of MPD on co-morbid anxiety, due to a larger effect on ADHD symptoms in patients with co-morbid anxiety compared to other patient conditions; unfortunately, placebo data were not available [127]. One placebo-controlled double-blind study showed that MPD improves emotional status in ADHD patients who do not have co-morbidities [128].

Given the high rate of co-morbid anxiety in ADHD and high risk of negative emotion in adolescents in general, the effect of MPD on the emotional dimension is of particular interest. Positive change in emotion has been reported as a qualitative effect associated with symptom reduction but not necessarily a direct effect of medicine [129]. Therefore more data on acute effects of MPD on emotion processing may be more illustrative.

Although MPD has been used to treat ADHD for more than 50 years with some degree of satisfaction, controversy exists regarding the exact actions of the drug. Doubt remains about whether drug-induced changes in one domain of function account for changes in other domains of function [130]. Examining acute effects of MPD in objective tests is particularly useful due to relatively precise event time points and control of factors. Although contradicting results remain, some evidence supports the hypothesis that MPD produces multi-faceted effects through multiple pathways.

Acute and chronic MPD treatment has been shown to reduce motor activity in children with ADHD, measured by recording with actigraph (a computer recording gross body and head movement)

[130-132]. However, these effects were situational, in line with the context-dependent expression of hyperactivity in ADHD [14-16;131].

MPD treatment also positively affects performance on cognitive tasks. These include working memory (e.g., measured by delayed spatial working memory task) [133-137], paired-association learning [138], sustained attention (measured by continuous performance test) [139], inhibitory control (e.g., measured by go/no-go task) [78], executive planning (prospective coding, e.g., measured by Tower of London) [135], and set-shifting (e.g., measured by attention set-shifting task) [135;140].

In contrast to the widely studied effects of MPD on activity and cognition, only a few groups have investigated effects of MPD on emotional processing using computerized testing. These data indicated a normalizing effect of MPD on emotional processing, especially for negative stimuli. One study showed that MPD normalized event-related potential to negative stimuli in ADHD [141]. In adolescents with ADHD performing an emotional Stroop task, acute MPD reduced error rates under negatively-valenced distraction, but had no effect on neutrally-valenced distraction [142]. Another study reported that MPD normalizes emotion-modulated startle response in adult ADHD but not in subjective rating of arousal and valence of stimuli [143].

These multifaceted effects of MPD in ADHD generate debate over how and where MPD works in ADHD. Does MPD strengthen the PFC inhibitory influence over subcortical areas, or act in parallel on the PFC and subcortical areas to normalize neural activity? Behavioral data suggest the latter, because the drug seems to more consistently improve inhibitory control and vigilance (e.g., regulated by PFC and striatum) [144-146] than working memory and planning (regulated primarily by PFC) [146-151]. However, what all of these studies do suggest is that additional investigation into MPD mechanisms of action is needed to develop a better understanding of its efficacy.

The Neurochemistry of Methylphenidate

As mentioned earlier, a number of catecholamine-related genes are significantly associated with ADHD [reviewed in 100;152;153] . These include genes encoding the DA receptors (D4 and D5), the DA transporter (DAT), and the DA beta-hydroxylase [reviewed in 154;155]—which is necessary for conversion of DA to NE [2]—and, most recently, genes that encode the NEergic transporter (NET) and D1 receptor [101;156-158]. A common hypothesis of the current research on neurochemistry of MPD, and of ADHD, is that DA and NE play significant roles in the disorder and its treatment, and that the drug affects extracellular levels of both neurotransmitters.

MPD binds to the transporters of DA and NE and inhibits reuptake of DA and NE in the brain [159-161]. Studies in healthy humans showed that MPD increases extracellular DA levels in the striatum [162;163]. Not surprisingly, pretreatment with haloperidol, a DA D2 receptor antagonist, blocks the improvement on vigilance tasks induced by MPD in hyperactive boys [164].

In line with a multifaceted effect of MPD, DA and NE modulate a wide set of functional systems in the brain. These include motor function, cognition, and emotion, all of which have been extensively implicated in the neurochemical basis of ADHD **(Fig. 2).**

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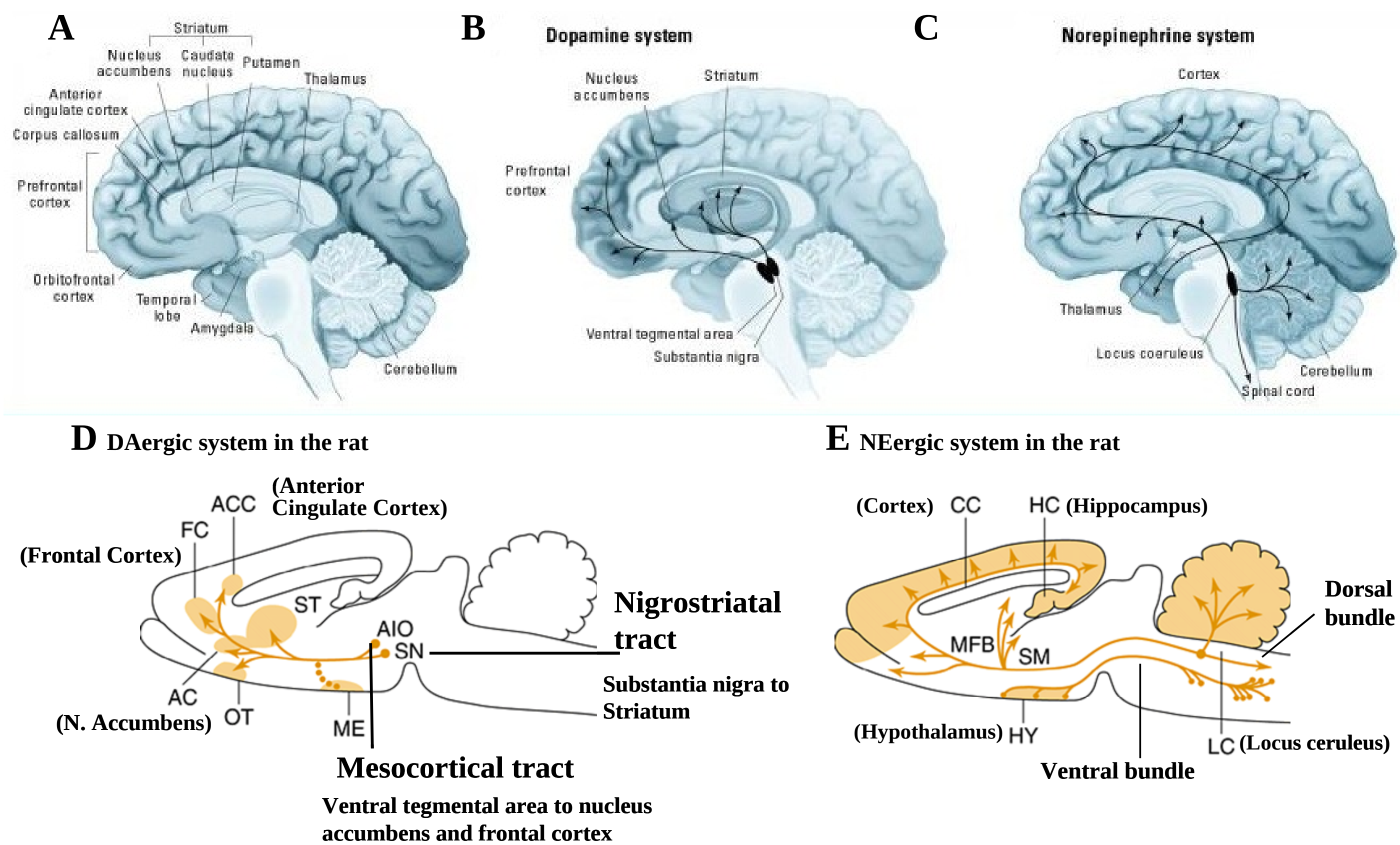


Figure 2. Illustration of the dopaminergic system and norepinephrine system in human and in rat, adapted from Bear et al., 2001, *Neuroscience: Exploring the Brain* [1] **and Kuhar et al., 1999, *Basic Neurochemistry*** [2]**. Upper panel:** The human brain, **A)** Mid-sagittal view showing major cortical and subcortical regions; **B)** Two groups of DAergic pathways: mesocortical and nigrostriatal tracts; **C)** Major NEergic pathways, the dorsal and ventral bundles. **Lower panel:** The rat brain, **D)** DAergic pathways: mesocortical and nigrostriatal; **E)** NEergic pathways, the dorsal and ventral bundles.

The DAergic system can be divided into two groups **(Fig. 2B, D).** The mesocortical DAergic tract mediates primarily locomotion, goal-directed behavior, cognition, and affect/emotion. The pathway starts in the ventral tegmental area and sends projections to the forebrain, including the frontal and cingulate cortex, septum, hippocampus, nucleus accumbens, and amygdala [2;165;166]. Nigrostriatal DAergic tract regulates largely reward-related functions [reviewed in 167]. The pathway originates in the substantia nigra and sends dense axons to the caudate nucleus and putamen of the striatum [2].

The NEergic innervations play a central role in cognition and emotion, as well as learning and memory—including that of movement and reflex mediated by the cerebellum [168-172]. This system is divided into two groups as well (**Fig. 2C, E)** [2]. The dorsal bundle has two components, both originating in the locus ceruleus. However, one projects to the spinal cord and cerebellum. The other projects anteriorly through the medial forebrain bundle to the entire cerebral cortex and structures such as amygdala, hippocampus, thalamus, and hypothalamus. The ventral bundle sends projections to the hypothalamus and brainstem.

The PFC and subcortical structures possibly all participate in the actions of MPD in the process of normalizing function and behaviors. Imaging studies on the effects of MPD found changes in activity in a wide range of structures, but with a seemingly selective role of subcortical areas in ADHD. MPD upregulates PFC activity to similar extents in both children with ADHD and healthy children [78]. In contrast, the drug upregulates striatal, hippocampal, and thalamic activity during tasks in affected children [78;173;174], but reduces or does not affect striatal activity during these tasks in healthy children [78;173]. More interestingly, MPD fails to normalize behavior when striatal activity is not normalized after drug administration, even though PFC activity is increased [137]. These data suggest that the actions of MPD in the subcortical and limbic areas contribute to the therapeutic effects of the drug on behaviors.

Contribution of Animal Studies to Understanding the Actions of MPD

While the human data point to a multipathway nature of ADHD and participation of the subcortical areas in the effects of MPD, animal studies have been able to unify the symptoms of ADHD and the effects of MPD using a single core structure.

The most recent focus of animal studies of MPD is the PFC. Rodent studies indicate that the reciprocal relationship between the PFC and striatum underlies the therapeutic effect of MPD on motor activity. As reviewed by Sullivan et al. [175], depletion of DA in the rodent PFC can upregulate striatal DA activity; restoring DA transmission in the rodent PFC normalizes striatal DA activity. For example, PFC DA depletion or receptor blockade enhances the responsiveness and activity of DA terminals in dorsal or ventral striatum [176-181]. On the other hand, stimulation of the DA receptors in the PFC reduces DA turnover in the dorsal striatum [182]. More interestingly, Sullivan et al. in the same review also noted that intracortical injection of DAergic agonists or DA releasing agents reduces motor activity. In particular, injection of amphetamine into the medial PFC inhibits the increase in locomotor activity induced by intra-accumbens injection of amphetamine, which is enhanced by blockade of DA D1 receptor in the PFC but not by inhibition of other DA receptor subtypes or NEergic and serotonergic receptors [183]. These data suggest that MPD may suppress hyperactivity in ADHD by elevating medial PFC DA levels, which, in turn, activates D1 receptors.

In assessing mainly rodent and non-human primate data, Arnsten and colleagues [184] hypothesized that MPD enhances PFC function by increasing DA and NE, which, in turn, stimulate DA D1 receptor and NE alpha-2A receptor, respectively. They also suggest that "too little or too much DA or NE impairs PFC function" [185]; that is, DA and NE modulate the PFC function with inverted-U-shaped dose-response curves. Supporting Arnsten's theory, in rats and monkeys, systemic administration of DA D1 antagonist or NE alpha-2 antagonist blocks the MPD-induced improvement in a PFC-dependent cognitive task [185;186]. Also, MPD at therapeutic doses increases extracellular levels of DA and NE in rat PFC, as well as levels of NE in rat hippocampus, but does not alter extracellular levels of DA in striatum [187].

Additionally, one argument supported by animal studies is that NE plays a predominant role in the action of MPD and the etiology of ADHD. Studies in adult rats [188] showed that MPD increases cortical cell excitability in PFC slices. Application of an alpha-2 noradrenergic antagonist blocked the MPD-induced increase in cortical excitability but application of a DA D1 receptor antagonist did not.

Indirect evidence from monkeys showed that infusion of yohimbine (NE alpha-2-receptor antagonist) directly into the PFC increases locomotor hyperactivity [189] and impulsivity [190], similar to lesions of the same area. Infusion of yohimbine into prefrontal cortex also weakens working regulation of memory and attention [191].

However, controversy remains. Some hypotheses emphasize that MPD acts through the subcortical DA system, as reviewed by Engert et al. and Levy [192;193]. Seeman and Madras [194;195] sought to explain the suppressive effect of MPD on hyperactivity. They hypothesized that DAT blockade increases extracellular levels of DA and activates the presynaptic DA D2 autoreceptors. Activation of autoreceptors decreases DA release in response to a stimulus and therefore reduces behavioral response to that stimulus. In line with this hypothesis, low doses of MPD decrease locomotor activity in the rat under an arousing condition [196]. A second hypothesis, proposed by Volkow and colleagues, explains the actions of MPD from a motivational perspective [197]. They suggested that the DAT blockade increases extracellular levels of DA despite the inhibitory effects of autoreceptor activation, which leads to accumulation of DA and, hence, amplifies DA signals. To support their hypothesis, Volkow and colleagues demonstrated in healthy participants that oral MPD increased striatal DA only when the subjects were presented with food stimuli [198] or were performing a monetarily-rewarded task [199], compared to neutral stimuli or a neutral task, respectively. More interestingly, the striatal DA levels correlated with subjective rating of how "hungry" the participants were for the food and how "motivating" the participants were for the task.

Where Animal Studies on MPD Fall Short: Emotion-related Function and Age-Specific Effects

While animal studies have uncovered much of the mechanics of MPD in the brain and its effects on behavior, animal studies have not specifically addressed the effects of acute MPD on emotion-related function. Only indirect evidence indicates a DA mechanism through the PFC modulation of subcortical and/or limbic systems [200]. For example, in urethane-anesthetized rats, local iontophoretic application of DA in the PFC attenuated the suppression PFC neuron firing that was induced by stimulation of the basolateral amygdala [201]. Further, DA depletion in the medial PFC decreased time spent in the open arms and enhanced protected risk-assessment behaviors, indicating that PFC DA participate in mediating anxiety-related behaviors [202].

Additionally, the PFC-centered model arises from adult animal studies, while the human data are derived mostly from pediatric patients. The brain during childhood and adolescence (juvenile period and adolescence in animals) undergoes developmental changes and differs from the adult brain in many aspects. Spear and colleagues, in an excellent review, suggested that the relative balance between subcortical and cortical function experiences a shift during adolescence [203]. These differences highlight the need for investigation of the actions of MPD in animals at different developmental stages.

From the areas where data are lacking, a specific question emerges: does MPD affect activity, cognition, and emotion in the same way throughout development, or differentially across ages? More precisely, are the three domains of effects manifested through a single pathway, as suggested by the adult data, or through separate structures?

The PFC in human and in mammals does not reach structural and functional maturation until late adolescence [204-206]. For example, the volume of PFC in humans [207-209] and in rats [210] declines around adolescence. In non-human primates, the number and density of cortical synapses peaks between 2 and 4 months postnatally, then slowly declines over a period of years to stable adult levels

[205]. In humans, working memory and cognitive flexibility keep improving from age 4 to 13 years [211;212]. Children of 7–12 years demonstrate greater volume of activation in the PFC during a go/no-go task than young adults of 21–24 years of age [213].

For both humans and animals, the subcortical and limbic systems experience a general peak of function around the onset of adolescence, compared to that in younger age or in adulthood. Both behavioral and functional imaging data suggested such developmental peaking and decline in the structures mediating locomotion and anxiety as well as learning and memory. Rats display low exploratory activity around the age of PND 25 and become robustly active after PND 28 [214;215]. In the emotional domain, the amygdala was activated when identifying emotional state from facial expressions, more so in adolescent humans than in adults [216;217]. In rats, an unfamiliar environment does not affect social interaction, an anxiety-related behavior, at PND 28 but decreases this behavior at PND 35 [218]. Meanwhile, the hippocampus, mediating learning and memory as well as anxiety, demonstrated responses to novelty in PND 30 not at PND 16 or PND 23 when using c-fos expression as an indicator of activation [219]. Preadolescent rats learn a spatial task slower compared to adolescents [220;221].

On the neurochemical level, the DA and NE systems are still developing throughout the pre- and peri-adolescent period. In non-human primates, DA fiber density in the PFC peaks during adolescence [222-224]; DA varicosities in the PFC reach and maintain peak values during early pubertal ages, then decrease dramatically until the end of puberty [225]. Rats also show peak DA fiber density and peak DA concentrations in PFC around PND 35 (or between 4 and 6 weeks) [226;227]. In contrast, the NE concentration in most cortical regions of the monkey brain increases at a slower rate (compared with

DA) and reaches the adult level in mid-puberty [220;221]. Therefore, the relative balance of DA and NE changes over the course of development.

Meanwhile, the subcortical areas undergo similar developmental changes in DA. Human striatum is reported to lose about 30 to 50% of DA D1-like and D2-like receptors during the period from juvenile to adulthood [228;229]. In rat striatum, D1 and D2 receptor binding on PND 40 are about 50% and 35% higher than those seen on PND 25 and during PND 60-120 (adulthood) [230-232]. The same study also reported an increase of approximately 150% in D1 and D2 receptor binding in rat nucleus accumbens between PND 25 and PND 40 [232]. Two other studies showed that D1 and D2 receptor binding declines by about 30% between PND 35 and PND 60 in both the striatum and nucleus accumbens [230;231]. Finally, adult levels of DAT binding in the striatum are reached by early-mid adolescence (PND 28–35) [233].

These developmental variations, therefore, suggest that the response to MPD, both behaviorally and neurochemically, may change throughout pre- and peri-adolescence and may differ from that in adulthood. Further, a period may exist during pre- and peri-adolescence when subcortical structures play more important roles in the actions of MPD. Indeed, compared to adult rats, juvenile rats respond to a lesser extent to MPD as measured by cellular events in the prefrontal cortex [234]. Information on the actions of clinically-relevant doses of MPD in the developing brain is needed to comprehend the animal studies in adults and bridge the pre-clinical and human research.

Investigations to Bridge the Gap

Given the numerous changes occurring across the brain during childhood and adolescence, concern exists that repeated exposure of MPD may cause lasting alterations of the brain. Children with ADHD are reported to show higher rates and earlier onset of drug abuse than unaffected individuals

[235-239]. On contrary, other reports, including a meta-analysis [240], indicate that treatment with MPD significantly reduces the risk for substance abuse in adolescence and adulthood [241-243].

Animal studies have identified a similar long-term "protective" effect of MPD against substance abuse, but also indicated that it is, in fact, the result of a depression-like change. Repeated administration of MPD during pre-adolescence (PND 20-35) in rats decreases adulthood preference to natural rewards and drug rewards while increasing corticosterone levels during restraint stress, compared to saline-treated controls [244;245]. These data are consistent with depression-like behaviors. Additionally, repeated exposure of MPD from PND 35-56 produces impairment in recognition memory of objects in adulthood [246]. While these studies were carried out in normal rats, concerns remain for treating children and adolescents with MPD.

In addition to treating ADHD, MPD is routinely used for several clinical conditions [247;248] and sometimes by healthy people as a "brain booster" [for a review, see 249]. *Nature* magazine conducted an online survey and found that about 12% of the 1400 responding readers have used MPD for non-medical reasons [250]. The United States prescribes far more Ritalin in comparison to other countries, suggesting the possibility that the medication is being over-prescribed [251].

Appropriate animal studies can contribute to better understanding and optimization of usage of MPD in children and adolescents. In animal studies it is easier to control for the influences of factors such as diet, compliance, environment, and similarity between subjects. The complex interactions involved in MPD treatment for ADHD makes control of these variables essential.

Preliminary Study

MPD affects cognition, activity, and possibly emotion in ADHD and in healthy humans [136;252;253], who take the drug orally. The acute and long-term effects of MPD are highly dose-dependent

[186;246;254;255]. Oral administration of MPD produces plasma levels with low peak values and less steep slopes, contrary to intravenous and intraperitoneal (i.p.) injections [256;256-259]. Additionally, in animals, handling during injections produces stress, which acutely increases extracellular NE in brain regions [260;261]. Therefore, low doses of MPD and oral administration are necessary to simulate the use of MPD in humans.

To begin bridging the gaps in our understanding of the mechanisms of MPD action, our preliminary study investigated the effects of voluntary oral ingestion of MPD at low doses on cognitive performance, locomotor activity, and anxiety in pre- and peri-adolescent rats (detailed methods and results can be found in our previous publications [262;263]) . To simulate the therapeutic pharmacokenitics of MPD, we administered the drug solution on a small cracker, such that rats ate it without handling. Testing and dosing occurred during the active phase of the rat's circadian cycle since circadian cycle influences response to MPD [264]. Others have shown that i.p. injection of MPD at 0.6 mg/kg in rodents during late dark phase does not increase locomotor activity, in contrast to injections during light phase and early dark phase [265;266].

We defined the period from PND 22 to 42 as pre- and peri-adolescence, as a rough equivalence to childhood and early adolescence (preschool-age and school-age) in human. In rats, PND 28-42 is defined as adolescence [reviewed in 203] and PND 20-35 is considered equivalent to 4-12 years in humans [reviewed in 267;268].

Preliminary Experiment 1. Age-Dependent Pattern in the Effect of MPD on Performance in Cognitive Tasks

Radial arm maze task was used to examine the effect of MPD on spatial learning and working memory [269]. Our preliminary study found that oral MPD at 3 mg/kg improved performance on a spatial learning and memory task with apparent age-dependent patterns. Treated rats scored higher than controls on performance during PND 22-24 but lower during PND 32-34 (**Fig. 3**).

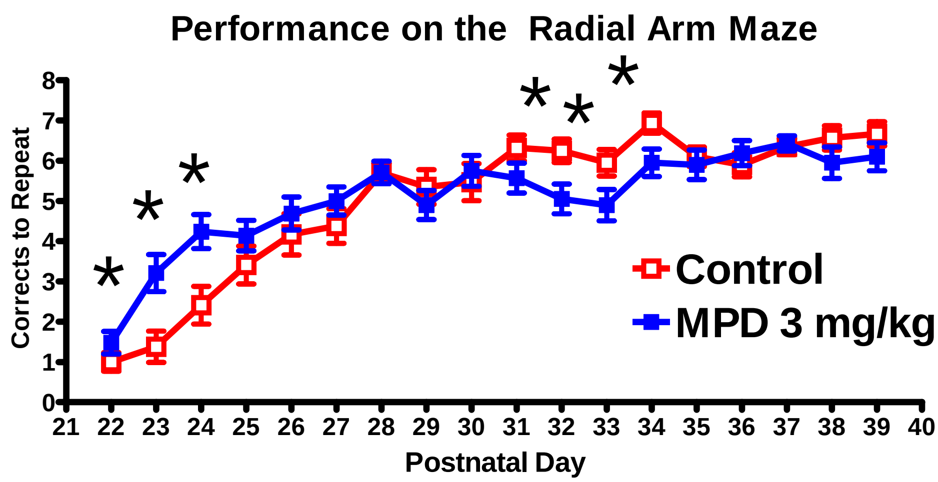


Figure 3. Oral administration of MPD at 3 mg/kg improved performance from PND 22 to PND 24 but impaired performance from PND 32 to PND 34 on the radial arm maze. (N=29/treatment group) * Significant difference from the control.

Similarly, an attention task from PND 22 to 43 showed age-dependent effects of MPD. In the task, rats learned to discriminate between two boxes to retrieve a food reward. The outsides of boxes were covered with cue materials, that is, black rubber or sandpaper. The black rubber was consistently associated with food reward. Three testing phases examined simple discrimination (cue materials covering the sides of the boxes, Training Phase), set-shifting attention (cue materials covering the lids, Set-Shifting Phase), and focused attention (novel task-irrelevant materials covering the sides of the boxes while cue materials covering the lids, Distraction Phase) [263;270]. Once a rat reached a criterion in a phase, it entered the next phase regardless of age. Therefore, rats started the Set-Shifting Phase and the Distraction Phase at different ages. MPD only helped the performance during Set-Shifting Phase for rats that rapidly proceeded through the simple discrimination phase (**Fig. 4**). Both control and treated rats that spent more trials during simple discrimination phase accomplished the following Set-Shifting Phase within a similar number of trials.

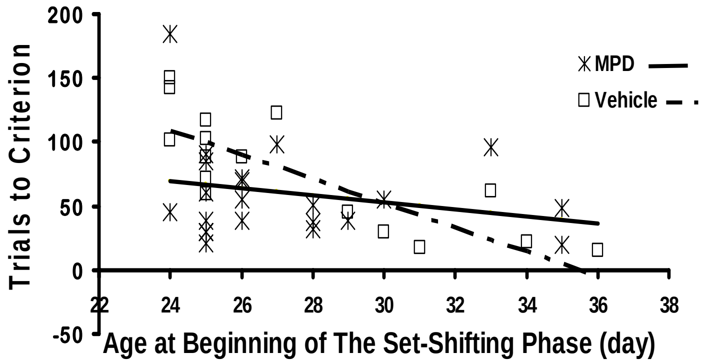


Figure 4. Trials to Criterion of each rat is plotted against that animal's age at the beginning of the Set-Shifting Phase. (N=16/treatment group) MPD reduced Trials to Criterion when age differences were controlled ($p<0.05$). MPD interacted with age since the drug only improved performance in animals that started the Shift Phase early ($p<0.05$).

Together, data from the radial arm maze and attention tasks suggest that MPD is more effective before a certain level of maturity has been reached. A dose-response curve of drug effects in a well-controlled test is necessary to critically test this hypothesis and to more clearly demonstrate the age-dependent pattern of effects on cognitive performance.

Preliminary Experiment 2. The Effects of MPD on Activity and Anxiety-Like Behavior in Weanling Rats

In a preliminary study on elevated plus-maze we found that oral MPD at 3 mg/kg acted as an anxiolytic drug (Fig. 5) PND 22. Rats treated with oral MPD at 3 mg/kg decreased stretch-attend postures and increased head dips, both of these findings are consistent with actions of an anxiolytic-like drug [271;272]. Tested in an activity recording box on PND 23, oral MPD at 3 mg/kg increased locomotor activity (Fig.6). The alteration of anxiety-like behaviors and locomotor activity by MPD could contribute to improved performance on the radial arm maze. Data at other ages are needed for a full profile of the effect of MPD on activity and anxiety-like behaviors during pre- and peri-adolescence.

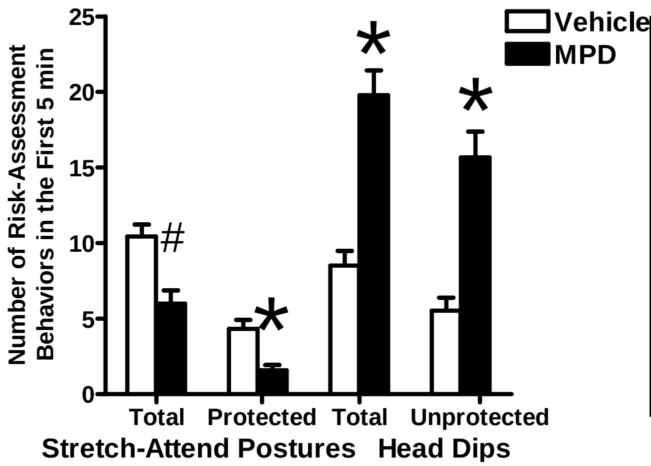


Figure 5. Effect of oral MPD on risk-assessment behaviors during the first 5 min on the elevated plus maze on PND 22. (N=16/treatment group) Mean numbers (+SEM) of stretch-attend postures, protected stretch-attend postures, head dips, and unprotected head dips are shown for the treatment groups. * Significant difference from the control ($p<0.05$). # Marginally significant difference from the control ($p=0.0644$).

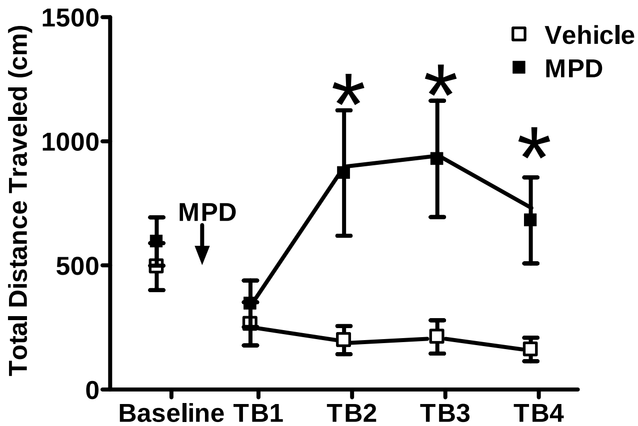


Figure 6. Effect of oral MPD on locomotor activity on PND 23. (N=16/treatment group) From 10 min to 40 min after dosing, that is, time blocks (TB) 2 through 4, the MPD-treated group traveled more distance than did the vehicle-treated group, with a peak of activity approximately 30 min (TB3) after dosing. * Significant difference between the vehicle group and the MPD-treated group ($p<0.05$).

In the activity recording test on PND 23, MPD also increased locomotor activity in rats that had higher baseline activity, but had minimal effects in rats that had lower baseline activity (Fig. 7) [263]. Baseline activity levels were collected during the first 15 min of exposure to the recording box. Since the recording boxes were novel to the rats, baseline activity levels could be considered as locomotor responses to novelty. Animal studies have shown that rats that have high levels of locomotor response to novelty demonstrate greater locomotor response to psychostimulants [273;274]. To date, no animal study has investigated the possibility of a similar correlation between the response to novelty and the cognitive response to MPD. Tracking individual baseline activity levels may help monitor individual differences in tone of neural systems and predict the individual differences in performance responses to MPD in cognitive tasks.

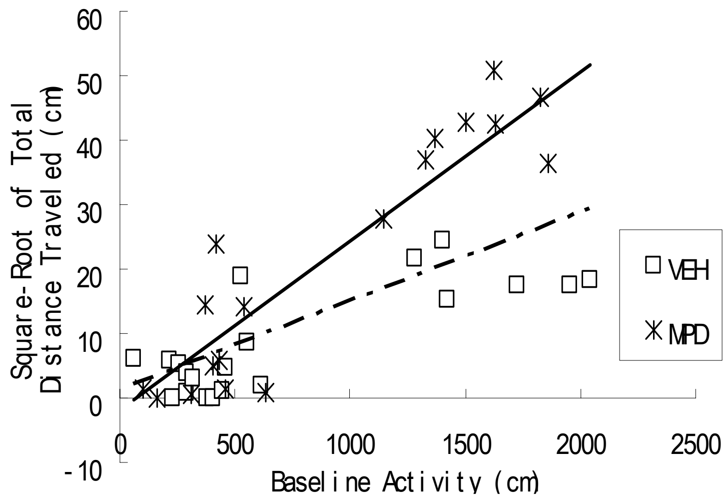


Figure 7. Individual locomotor responses to novelty predict locomotor responses to oral MPD on PND 23. (N=16/treatment group) Regression lines for the MPD-treated and control groups, show the relationship between baseline activity (distance traveled during the first 15 min in a novel environment) and the square-root of individual total distance traveled during the time period from 10 to 40 min after dosing. Slopes of the two regression lines were significantly different ($p \leq 0.001$).

From the preliminary data, we hypothesized that MPD produces multifaceted effects in pre- and peri-adolescent rats on cognition, anxiety, and activity, all of which contribute to improvement on performance in cognitive tasks. The constellation of these effects changes across ages. Individual response to novelty may also predict the effects of oral MPD on cognition, anxiety, and activity. Testing this hypothesis of age-dependent effects of MPD would improve our understanding of the drug in periadolescent animals and bridge the gaps in current knowledge of use of this drug in ADHD.

Experiments

The present study characterized the effects of MPD in pre- and peri-adolescent rats on cognition, anxiety, and activity, as well as whether the response to novelty would predict individual differences in response to MPD. First, radial arm maze tests spatial learning and working memory. Initial learning and performing of the task also requires low levels of anxiety and relatively high levels of locomotor activity. Second, elevated plus-maze measures anxiety-like behaviors as well as locomotor activity.

Brain and plasma levels of MPD were analyzed to detect possible differences in drug levels as a function of age. Before receiving any drug administration and behavioral testing, locomotor activity of each rat in all experiments was recorded for 15 min in a novel environment, i.e., baseline activity. Individual baseline activity was analyzed for correlation with individual behavioral measures from the radial arm maze and elevated plus-maze tests. Methods and materials, except data analysis, are summarized in the appendix entitled **"Supplementary Methods"** located at the end of this document.

General Rationale

Selection of Doses

Oral MPD doses of 2, 3, and 4 mg/kg were used to produce plasma levels within the range of therapeutic levels seen in humans. Therapeutic doses of MPD in humans typically yield peak plasma levels from 8 to 40 ng/ml [275]. In rats, 2 mg/kg and 3.5 mg/kg of oral MPD produces peak plasma levels of 36 ng/ml and 62 ng/ml 15 min after dosing, respectively [254]. Additionally, oral MPD within the range of 1.0 mg/kg to 3.0 mg/kg improves performance in cognitive tasks with individual differences [186;262]. Taking age and individual differences into account, 2, 3 and 4 mg/kg of oral MPD should produce plasma levels that are similar to those produced by therapeutic doses in humans.

Time Course

The present study examined three age groups of rats: PND 23 to 28, PND 29 to 34 and PND 36 to 41. These ranges were selected based on a set of standards including learning ability, activity level, and development of DA and NE systems, as summarized in **Table 1**.

Table 1. Developmental States during Pre- and Peri-adolescence in the Rat					
Developmental Changes		**Age 1 (PND 23-28)**	**Age 2 (PND 29-34)**	**Age 3 (PND 36-41)**	**Reference**
Cognition	Learning ability	Low	Intermediate	High	276
	Continuous oral MPD on radial arm maze	Improved performance	Impaired performance	No difference compared to the control	262
Anxiety	Corticosterone levels	High baseline, low response	Low baseline, high response	Not reported	277
Loco-motor	Exploration	Low	High	High	214;278
Neuro-chemistry	DA system in PFC and the subcortical areas	Low	Peak	Lower than Age 2	226;279
	NE levels and receptors	Relatively Low	Adult Level	Adult Level	280-282

Experiment 1. Radial arm maze task during 3 age ranges of pre- and peri-adolescence.

Hypothesis: Oral MPD will produce age-dependent effects on cognitive performance during pre- and peri-adolescence.

Rationale: Working memory is the ability to actively hold or manipulate information. . Performance in spatial working memory tasks is improved by MPD in both children with ADHD and typical individuals [135;136]. Working memory in humans keeps improving from age 4 to 13 years [211], and a similar developmental trajectory is reported in rodents [283]. **Preliminary experiment 1 (Figs. 3, 4)** demonstrated an age-dependent pattern for the effects of MPD on performance in cognitive tasks. We therefore hypothesized that oral MPD would affect performance on the radial arm maze depending on the stages of development (ages).

Win-shift radial arm maze task assesses spatial learning and working memory in rats[reviewed in 284;285;286]. "Win-shift" refers to the paradigm where each arm is baited only once in a given trial; the rat must shift to other un-visited arms to retrieve another reward. To retrieve all rewards with minimal effort, the rat has to retain and manipulate information about the arms of the maze to know which arms it has already visited.

The non-delay version of win-shift radial arm maze task utilized is hippocampus-dependent [reviewed in 287] and regulated by DA [288] system. Additionally, MPD reportedly affects hippocampal function *in vitro* through NE system [289]. Kuczenski and Segal [187] showed that oral MPD at 2.5 mg/kg significantly increased extracellular NE levels in the hippocampus in late adolescent rats. Although Kuczenski and Segal did not analyze the effect of oral MPD on extracellular DA levels in the hippocampus, NET is reported to uptake DA in the hippocampus [290] and has greater affinity for DA than does the DAT itself [291]. Therefore, oral MPD at a dose that elevated extracellular NE levels, that is, a sign of blockade of NET, may also reduce the reuptake of DA and result in elevated extracellular DA levels in the hippocampus. Hence oral MPD at clinically-relevant dose may enhance hippocampal function.

Highlights of Methods

All measures recorded during testing on the radial arm maze are summarized in **Table 2**. All secondary measures derived from direct measures are summarized in **Table 3**. A schematic (**Fig. 8**) summarizes the behavioral procedure for Experiment 1 (for detailed information of subjects, apparatus and behavioral procedure, please see **Supplementary Methods**). To compare the responses to MPD in pre- and peri-adolescent rats to adult rats, 81-day old male rats were tested on the radial arm maze following the same dosing and testing procedure.

Figure 8. Design of Experiment 1.

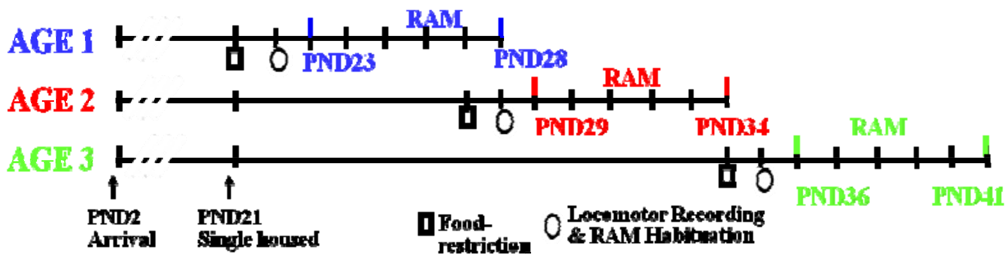


Table 2. Measures Taken during the Radial Arm Maze.			
Category	**Dependent Measures**	**Description**	**Relation with Function**
Accuracy	*Corrects to Repeat*	Number of correct choices a rat made before it revisited an arm that it had already explored for the first time.	Positive
	Error in 8 Entries	Number of revisiting errors a rat made in the first 8 entries.	Negative [b]
Movement	*Total Entries*	Number of entries a rat made in a trial.	Positive [a]
Efficiency	*Time to 8 Entries* [c]	Time for a rat to make 8 entries, in seconds.	Negative
	Time to 8 Arms	Time for a rat to visit all 8 arms, in seconds	Negative
	Time to Finish	Time for a rat to retrieve all 8 baits, in seconds	Negative
	Latency to leave the center	Latency before the four limbs of a rat first leave the center, in seconds	Negative

[a] Positive relation indicates that the higher score a rat obtained, the better it performed.
[b] Negative relation indicates that the lower score a rat obtained, the better it performed.
[c] The word *entry* refers to an arm-entry movement regardless of accuracy; the word *arm* refers to an entry to an arm that was explored for the first time in a trial.

Table 3. Secondary Measures in the Radial Arm Maze Test.			
Category	**Dependent Measures**	**Description**	**Relation with Function**
Accuracy	*Modified Corrects to Repeat*	*Corrects to Repeat* excluding rats that made 3 or fewer total entries and did not commit an error in a trial	Positive [a]
Strategy	*Strategy Score*	$\delta/\lvert\mu\rvert$ of the frequencies of choosing each of the 8 possible moves.	Foot Note [b]
	Strategy in 8 Entries	Within the first 8 entries, the $\delta/\lvert\mu\rvert$ of the frequencies of choosing each of the 8 possible moves	Foot Note [b]
Efficiency	*Time Per Entry in the first 8 Entries*	Time for a rat to finish an entry in the first 8 entries, in seconds (Time to 8 entries divided by 8) [c]	Negative [d]

[a] Positive relation indicates that the higher score a rat obtained, the better it performed.
[b] The lower the score, the more evenly distributed (random) the entry choices were.
[c] If a rat made fewer than 8 entries in a trial, Time Per Entry equals Time Per Entry in the first 8 Entries.
[d] Negative relation indicates that the lower score a rat obtained, the better it performed.

The *Strategy Score* was calculated using a published method [292]. **Fig. 9** shows illustrated examples of Choice Types. **Fig. 10** represents four sample animals to illustrate the relationship between Strategy Score and actual patterns of behaviors; more biased selection of Choice Type generates higher *Strategy Score*.

(Figures on the next page)

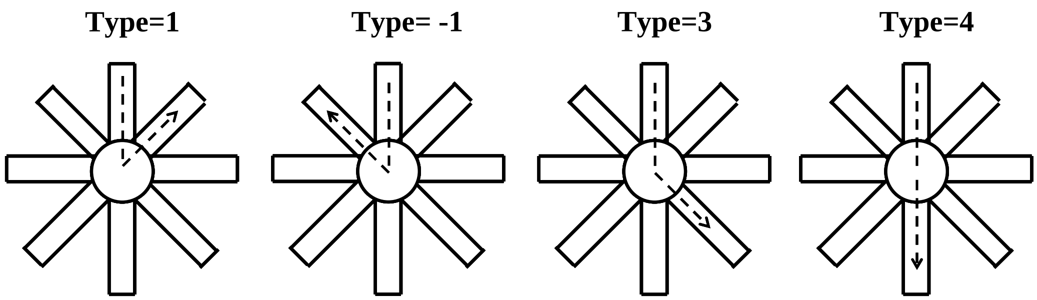


Figure 9. Examples of choice types in calculating *Strategy Score*
Clockwise turning to the adjacent arm was assigned as **Type 1**. Counter-clockwise turning to the adjacent arm was **Type -1**. Clockwise skipping 1 and 2 arms were assigned as **Type 2** and **Type 3**, respectively. Counter-clockwise skipping 1 and 2 arms were assigned as **Type -2** and **Type -3**, respectively. Entering the opposing arm was assigned as **Type 4**.

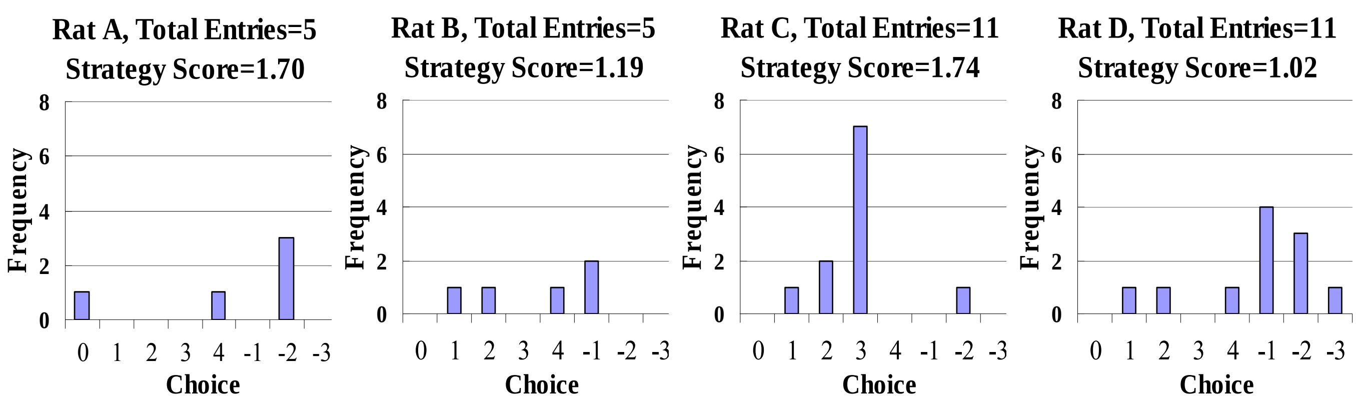


Figure 10. Sample animals showing the relationship between *Strategy Score* and patterns of behaviors Given the same total number of entries in a trial (Total Entries), more biased selection of Choice Type generates higher *Strategy Score*.

Data Analysis

Total Entries and *Errors in 8 Entries* of all four age groups were analyzed together in generalized mixed linear models. Both dependent variables were modeled as binomially-distributed dependent variables. Fixed factors included day, age, and treatment. Litter was a random factor. Baseline activity level and daily bodyweight change were linear covariates.

For *Corrects to Repeat*, the three pre- and peri-adolescent age groups were analyzed together; adult rats were analyzed in a separate model. Generalized mixed linear models were applied for each analysis. *Corrects to Repeat* was modeled as binomially-distributed dependent variable. Fixed factors included day, age (if applicable), and treatment. Litter was a random factor. Baseline activity level and daily bodyweight change were linear covariates. A compound symmetry structure was modeled for the within-subject covariance matrix. Satterthwaite corrections were applied to denominator degrees of freedom. We conducted additional analyses using the aforementioned parameters over the first 2 testing days of each age group, since the preliminary radial arm maze study showed significant differences between the treated and the control groups within the first 2 testing days.

Continuous variables were analyzed using mixed linear models. Fixed factors included day, age (if applicable), and treatment. Litter was a random factor. Baseline activity level and daily bodyweight change were also controlled as linear covariates.

Results

Movement

For illustrating purpose, figure of *Total Entries* is shown in the appendix entitled "Supplementary Figures" located at the end of this document. *Total Entries* revealed significant age ($F[3, 51.43]=43.55$, $p<0.001$; **Supp**. **Fig. 1A**) and treatment effect ($F[3, 51.43]=4.25$, $p=0.007$; **Supp**.

Fig. 1B), but not effect of day or interaction of age, treatment, and day (**Supp**. **Fig. 1C**). Older rats made fewer entries on the radial arm maze, with the adults rarely making any entry, especially in the first three days. Oral MPD at 4 mg/kg but not 2 and 3 mg/kg increased total number of entries when age groups and days were collapsed. Given that the adult group displayed a distinctive pattern of movement compared to that of the pre- and peri-adolescent groups on the radial arm maze, data from the two age ranges were analyzed separately in subsequent analyses.

Accuracy

Errors in 8 Entries revealed a significant effect of age ($F[3, 1.27]= 16.21$, $p<0.001$; not shown) and a significant interaction of age by day effect ($F[15, 0.72]= 2.61$, $p=0.001$; for illustrating purpose, shown in **Supp**. **Fig. 2A**). There was no significant effect of treatment and day, interactions of treatment and age, treatment and day, or interaction of treatment, age, and day (for clarity of the figure, showing in **Supp**. **Fig. 2B**). The pre- and peri-adolescent rats gradually made fewer errors in the first 8 entries as training proceeded whereas the adults gradually made more errors in the first 8 entries. Oral MPD at all doses did not significantly affect the number of errors rats made in the first 8 entries.

Corrects to Repeat in the pre- and peri-adolescent rats showed a significant treatment-by-age interaction ($F[6, 172.5]=3.09$, $p=0.0067$; **Fig. 10**, upper panel) and a significant day effect from Day 1 to Day 2 ($F[1, 126.7]=12.42$, $p<0.001$). Simple effects analysis stratified by age revealed significant differences among treatment levels in Age 3 (PND 36-37; $p=0.012$). Within Age 3, rats treated with MPD at 3 mg/kg and 4 mg/kg made significantly more entries before revisiting an arm, compared to the control group ($p=0.004$ and $p=0.027$, respectively) during the first 2 days. Rats treated with 2 mg/kg of MPD did not differ from the control ($p=0.093$). Adult (PND 81-82) performance on the radial arm maze revealed no significant effects of treatment.

We refined the dataset of *Corrects to Repeat* and generated *Modified Corrects to Repeat* by removing rats that made 3 (chance level performance) or fewer entries and did not commit an error in a trial. If a given rat met exclusion criteria in one trial but did not meet the criteria in another trial, data of this rat was only removed for the trial during which it met criteria. Observation during experiment suggested that a portion of rats in both the control and treated groups did not readily explore the maze, particularly during the first 2 or 3 days. Within this subgroup, a portion of rats made so few entries in a trial that they did not commit a revisiting error. In this condition, *Corrects to Repeat* could not exclusively represent how well the rats maintained information about the past experience but were presumably heavily influenced by locomotor activity and anxiety. On the other hand, data of a given rat remained in the dataset if the rat committed one or more errors regardless of the total number of entries it made in a given trial.

Modified Corrects to Repeat detected no significant difference between any of the treated groups and their age-matched controls (**Fig. 11**). **Fig. 12** summarizes the percent of rats removed according to criteria in each treatment by age group. More rats in the control group than in the treated groups did not readily move on the maze and were removed from the analysis, especially in Age 3.

Strategy

Fig 13 illustrates *Strategy Scores* of the pre- and peri-adolescent groups: there was significant effect of treatment ($F[3, 0.353]=3.673$; $p=0.015$). Rats treated with MPD at 4 mg/kg had lower scores compared to the control ($p=0.01$) across trials and ages. There was no effect of day or interaction of day, treatment, and age.

Since *Corrects to Repeat* was determined within the first 8 entries in a trial, *Strategy in 8 Entries* was calculated to further detect possible differences between the control and the treated groups in strategy utilization that contributed to the accuracy of performance. Interestingly, *Strategy in 8 Entries* of the pre- and peri-adolescent groups revealed no significant effect of treatment, age and day. No interaction of fixed factors was detected (**Fig. 14**).

The strategy data, combined with the accuracy data, suggest that rats treated with vehicle and oral MPD at 2, 3, and 4 mg/kg utilized comparable strategies to achieve similar levels of accuracy measured by *Corrects to Repeat* on the radial arm maze during pre- and peri-adolescence.

Measures of Efficiency

Measures of efficiency did not detect significant differences between any treated group and their age-matched control group. Only age effect or age by day interaction was found in these measures. For the purpose of better illustration, figures are shown in **Supplementary Figures**.

Rats aged PND 36-37 performed less efficiently on the maze compared to rats in Ages 1 and 2. Time to 8 Entries showed significant age by day interaction as rats in Age 3 took more time to make 8 entries, compared to rats in Ages 1 and 2, on Day 1, Day 4, and Day 6 (**Supp. Fig. 2**). Time per Entries in the First 8 Entries showed similar age by day interaction. That is, rats in Age 3 took more time to make an entry compared to rats in Ages 1 and 2 on Day 1 (**Supp. Fig. 3**). Time to Visit 8 Arms and Time to Finish showed similar but less prominent patterns (**Supp. Fig. 4, 5**). Latency to Leave the Center did not affect any fixed factor.

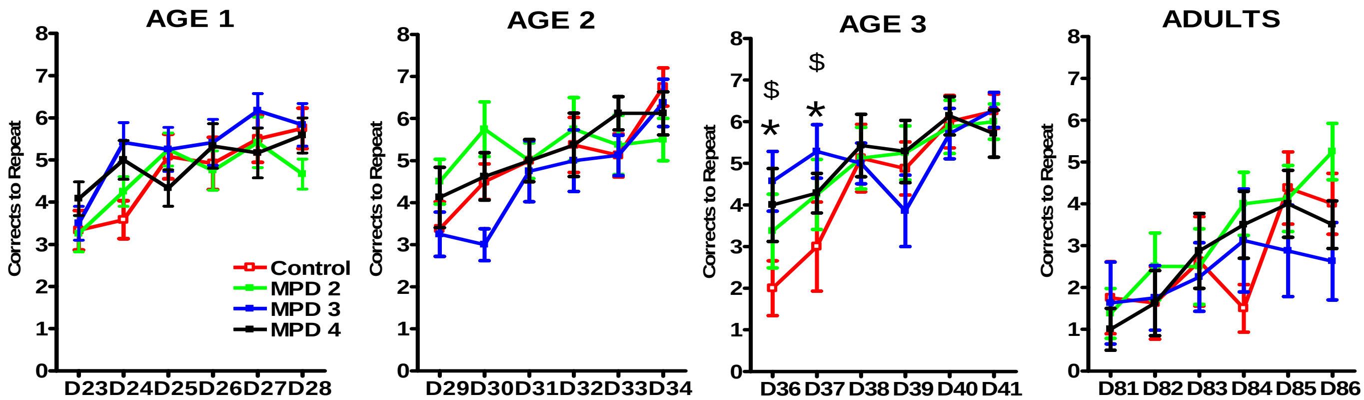


Figure 10. Effect of low doses of MPD on *Corrects to Repeat* on the radial arm maze. (N=12 or 8 per treatment by age group for Age 1 or all other age groups, respectively) Mean±SEM of *Corrects to Repeat* shown for treatment x age groups. $ Significance between Dose 3 and the control (P=0.004). * Significance between Dose 4 and the control (p=0.027)

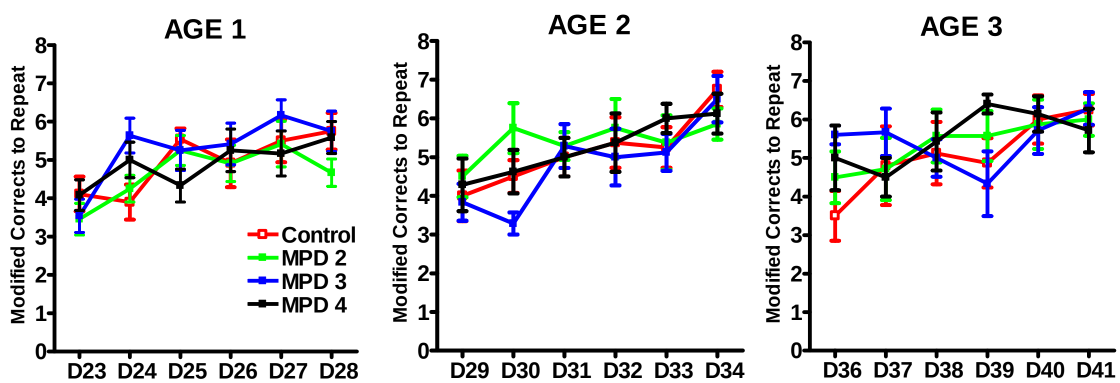


Figure 11. Mean±SEM of *Modified Corrects to Repeat* (N=12 or 8 per treatment by age group for Age 1 or all other age groups, respectively) No significant difference among treatment, age, or treatment by age groups.

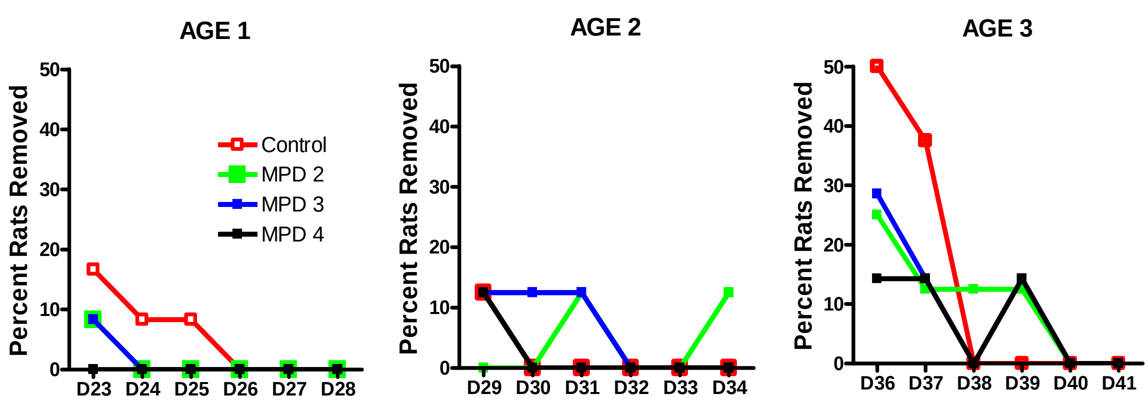


Figure 12. Percentage of Rats Removed in Calculating *Modified Corrects to Repeat* (Before removal of rats, N=12 or 8 per treatment by age group for Age 1 or all other age groups, respectively). More rats in the control group than in the treated groups did not readily move on the maze and were removed from the analysis, especially in Age 3.

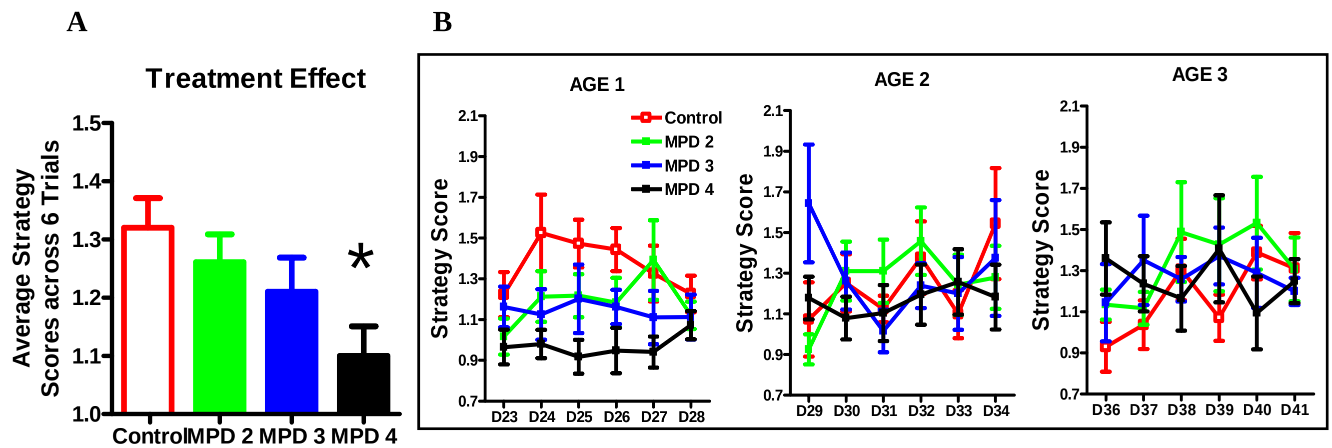


Figure 13. Mean±SEM of *Strategy Scores* (N=12 or 8 per treatment by age group for Age 1 or all other age groups, respectively) A: Treatment effect of low doses of MPD on the strategy used in the radial arm maze test, averaged across trials. * Significance between Dose 4 and the control (p=0.01). **B:** Strategy Scores of treatment by age groups across 6 days. No significant change of strategy score across trials.

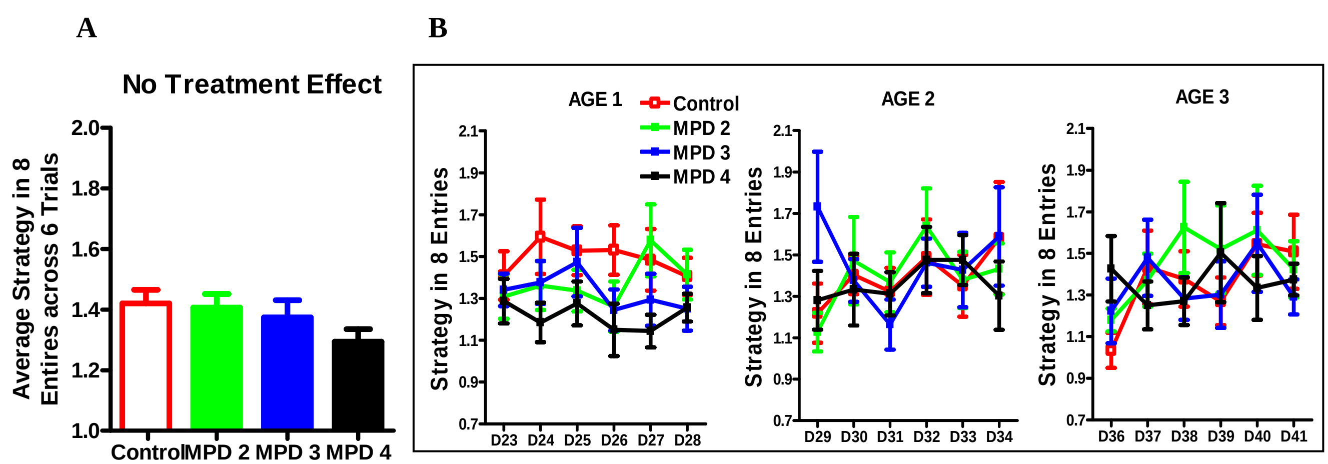


Figure 14. Mean±SEM of *Strategy in 8 Entries* across trials (N=12 or 8 per treatment by age group for Age 1 or all other age groups, respectively) A: No treatment effect in the strategy used in the first 8 entries, averaged across trials. **B:** Strategy Scores of treatment by age groups across 6 trials. No significant change of *Strategy Scores in 8 Entries* across trials

Summary

Oral MPD doses of 3 and 4 mg/kg improved performance on the radial arm maze during PND 36-37 but not PND 23-28 or PND 29-34. Combined over ages, oral MPD at 4, but not 2 or 3 mg/kg, significantly increased total number of entries compared to the vehicle control. Rats aged PND 36-37 performed less efficiently on the maze compared to rats in Ages 1 and 2. Oral MPD induced the improvement likely by facilitating the rats' movement on the maze as well as enhancing accuracy. On the other hand, oral MPD during pre- and peri-adolescence did not affect the number of errors rats made within the first 8 arms or the rats' strategy within the first 8 entries. Adult rats rarely entered any arm on the radial arm maze during the first several trials and demonstrated no difference in any behavioral measure among treatment groups.

Experiment 2. Elevated plus-maze in three age ranges of pre- and peri-adolescence.

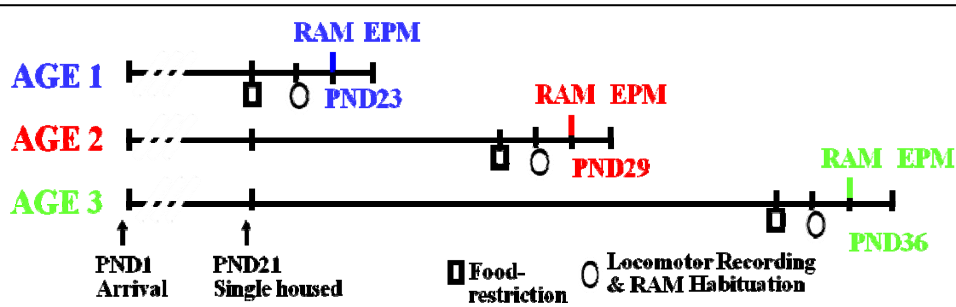
Hypothesis: Oral MPD will produce multifaceted effects of during peri-adolescence, of which the constellation changes across ages.

Rationale: MPD is reported to affect emotional processing in children and adults with ADHD. Both anxiety-like behaviors and locomotor activity influence performance on radial arm maze [269;286]. MPD altered anxiety and locomotor activity (**Preliminary Experiment 2**), which could have contributed to the enhanced performance of MPD-treated rats on radial arm maze.

The circuits mediating anxiety-related behaviors and locomotor activity are still developing throughout childhood and adolescence [203;212;278]. Consequentially, the effects of MPD on anxiety-related behaviors and locomotor activity may change during this period and, therefore, contribute differentially to the performance of MPD-treated subjects on cognitive tasks. A full profile of the effects of MPD on activity and anxiety-like behaviors during pre- and peri-adolescence will elucidate the possible contribution of the multifaceted effects of oral MPD to the effects on performance in cognitive tasks.

Additionally, anxiety plays a role in a variety of real-life activities and experimental tasks in humans and in animals [269;reviewed in 293;294;295;296]. Adolescence in humans is a high-anxiety period [reviewed in 203;297]. Understanding the effect of MPD on anxiety-like behaviors will help to understand the clinical use of MPD, and its activities in the brain, during this developmental period.

Figure 15. Design of Experiment 2.



Highlights of Methods

A schematic (**Fig. 15**) summarizes the behavioral procedure for Experiment 2 (for detailed information of subjects, apparatus and behavioral procedure, please see **Supplementary Methods**). **Table 4** summarizes the measures taken for anxiety-related behavior and locomotor activity on the elevated plus-maze [298; for reviews, see 299;300].

Data Analysis

Separate generalized mixed linear models were constructed for Open Arm Time (OA Time, anxiety), *Entries to Enclosed Arms* (Entries to EA, locomotor activity/exploration), *Grid Crossing* (locomotor activity), and *Percent of Protected Stretch-Attend Postures* (%PSAP, anxiety). Fixed factors were age (Age 1, 2, 3), treatment (0, 3). Litter and baseline activity level were controlled as a random factor and a linear covariate, respectively. Satterthwaite corrections were applied to denominator degrees of freedom.

<table>
<tr><td colspan="5">Table 4. Behavioral measures, terms, and descriptions used in the plus-maze test.</td></tr>
<tr><td colspan="3">Behaviors</td><td>Description</td><td>Correlation with anxiety</td></tr>
<tr><td colspan="3">Open Arm Time</td><td>Time spent in the open arms</td><td>Negative</td></tr>
<tr><td rowspan="3">Risk-Assessment Behaviors</td><td colspan="2"></td><td>More sensitive measures of anxiety on the elevated plus-maze than Open Arm Time</td><td></td></tr>
<tr><td rowspan="2">Stretch-attend postures (SAP)</td><td></td><td>The rat stretched forward to its full body length without moving the hind limbs and then retracted to its original position.</td><td></td></tr>
<tr><td>Protected SAP</td><td>SAP occurred in the "protected" areas.*</td><td>Negative</td></tr>
<tr><td colspan="3">Entries to Enclosed Arms</td><td>Number of entries to the enclosed arms</td><td>N/A, locomotor activity measure</td></tr>
<tr><td colspan="3">Grid Crossing</td><td>Number of gridlines on arms of the maze crossed by the subject</td><td>N/A, locomotor activity measure</td></tr>
<tr><td colspan="5">*The enclosed arms, the center platform, and the railed areas of the open arms were classified “protected areas”.
N/A: No relationship with anxiety</td></tr>
</table>

Results

Measures of Anxiety

Open Arm Time showed a significant treatment effect as the treated group for all ages spent more time in the open arms compared to respective control groups ($F[1,33.6]=17.58$, $p<0.0012$; **Fig. 15**). Percent of *Protected Stretch Attend Postures* revealed a similar significance in treatment effect ($F[1,35.6]=19.90$, $p<0.0001$). The group treated with oral MPD at 3 mg/kg in all ages showed a lower Percent of *Protected Stretch Attend Postures* (**Fig. 16**). No significant age, baseline activity effect, or interaction among factors was detected in Percent of *Protected Stretch Attend Postures*.

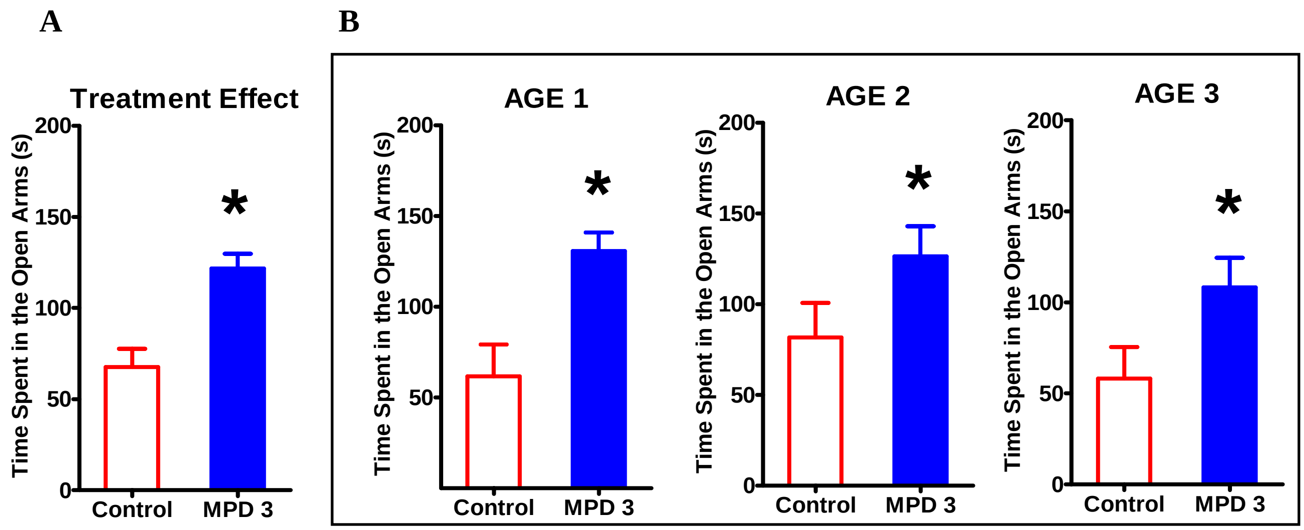


Figure 15. Treatment effect in time spent in the open arm (N=22/treatment group) Mean ± SEM of time spent in the open arms is shown for **A)** the treatment groups, collapsed across age; **B)** the age by treatment groups, showing no age difference in the treatment effect.. * Significant difference from the control.

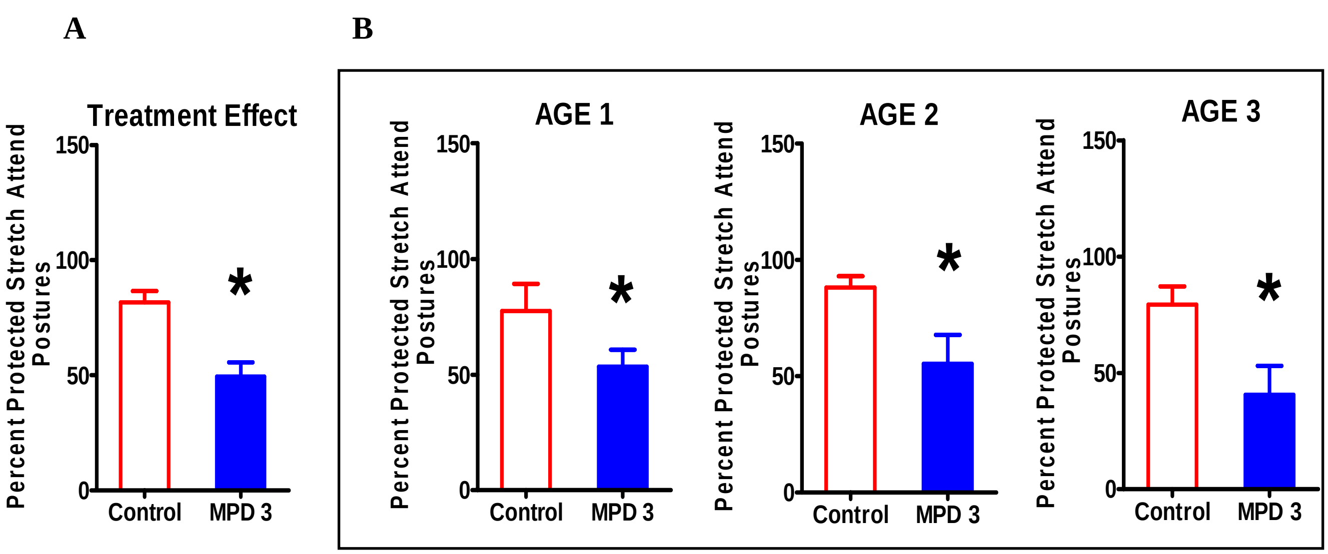


Figure 16. Treatment effect in *Percent of Protected Stretch Attend Postures* (N=22/treatment group) Mean ± SEM of percent of *Protected Stretch Attend Postures* is shown for **A)** the treatment groups, collapsed across age; **B:** The age by treatment groups, showing no age difference in the treatment effect. * Significant difference from the control.

Measures of Locomotor Activity

Entries to Enclosed Arms revealed a main effect for treatment ($F[1,25.8]=15.53$, $p<0.001$), age ($F[2,30.5]=6.27$, $p<0.01$), and a treatment by age interaction ($F[2,25.9]=5.99$, $p<0.01$). The effect of MPD in Age 1 (tested on PND 24) differed significantly from that in Age 2 (tested on PND 30, $p=0.002$) and in Age 3 (tested on PND 37, $p=0.006$). As illustrated in **Fig. 17,** the MPD-treated group in Age 1 made more *Entries to Enclosed Arms* compared to the control ($p<0.001$). In contrast, MPD treatment had no effect on number of *Entries to Enclosed Arms* for Ages 2 and 3.

Grid Crossing revealed significant treatment ($F[1,33.81]=30.50$, $p<0.001$), age ($F[2,38.13]=6.63$, $p=0.0034$), and baseline activity effects ($F[1,38.18]=14.15$, $p<0.001$), as well as a significant interaction of treatment and age ($F[2,34.01]=6.81$, $p=0.0033$) (**Fig. 18**). Simple effect analysis showed that the MPD-treated group in Age 1 ($p<0.001$) and 3 ($p=0.039$) made more *Grid Crossing* compared to the control but not those in Age 2.

Summary

On the elevated plus-maze, oral MPD at 3 mg/kg increased time spent in the open arms while decreasing the percent of protected stretch attend postures in all three ages. Oral MPD at 3 mg/kg also increased locomotor activity in Age 1 as indicated by the increased *Entries to Enclosed Arms* and *Grid Crossing* in the treated group. The treatment increased locomotor activity to a lower extent in Age 3 since *Grid Crossing,* but not *Entries to Enclosed Arms,* was elevated in the treated group.

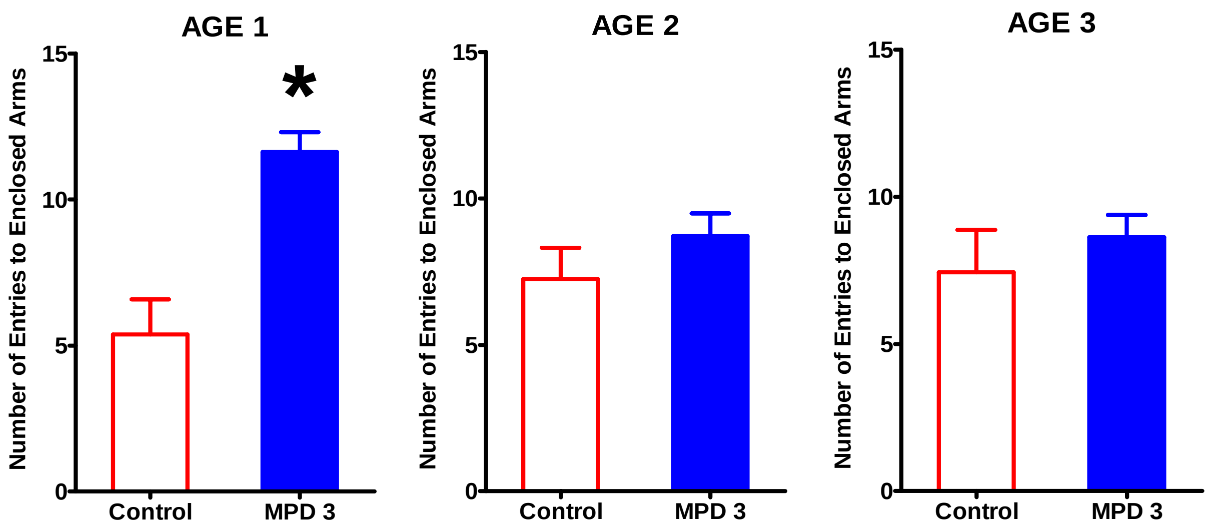


Figure 17. Treatment by age interaction in the number of entries to the enclosed arms (N=7-8/treatment x age group) Mean ± SEM of numbers of *Entries to Enclosed Arms* are shown for treatment by age groups. * Indicates significant difference from the control.

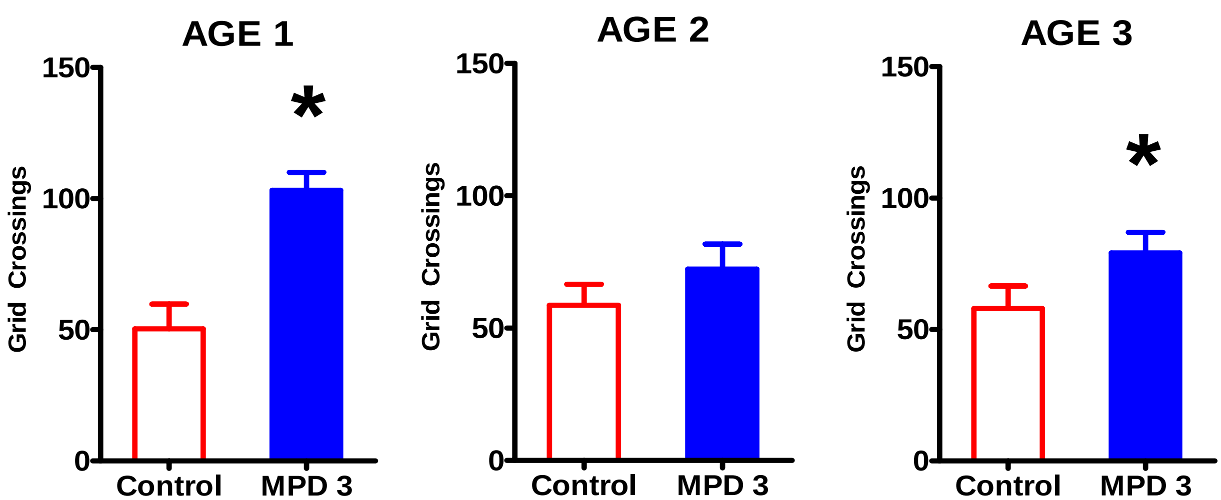


Figure 18. Treatment by age interaction in *Grid Crossing* (N=7-8/treatment x age group) Mean ± SEM of *Grid Crossings* is shown for each treatment x age groups. * Significant difference from the control.

Experiment 3. Brain and plasma levels of MPD in three age ranges of pre- and peri-adolescence.

Hypothesis: The absorption and metabolism of MPD will contribute to, but not fully account for, the age-dependent effects of oral MPD on behaviors.

Rationale: Brain and plasma levels of a drug provide fundamental information for understanding the effects of the compound. To our knowledge, brain and plasma levels previously have not been paired with behavioral responses during pre- and peri-adolescence.

Given the observations in experiments 1 and 2, we must rule out the possibility that differences in absorption, metabolism, and bioavailability of the drug produce the age-dependent drug effects on the behavioral performance. We chose to analyze the group receiving 3 mg/kg of MPD, at the ages when the most pronounced drug effects and age differences occurred.

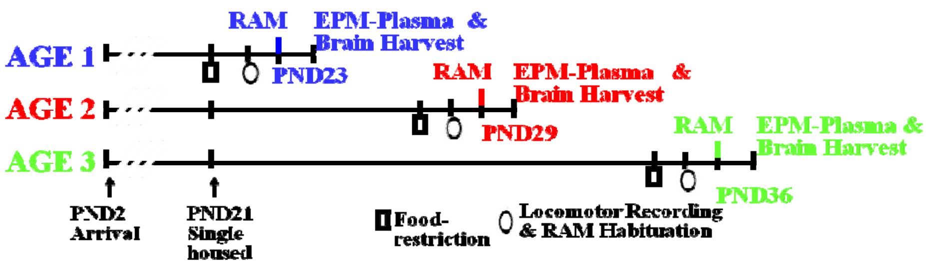
MPD undergoes extensive metabolism in the liver and blood in both humans and animals [258;301;302]. The predominant metabolites of MPD in the rat brain are ritalinic acid and para-hydroxymethylphenidate; these are measured at levels at least 10-fold lower than that of unaltered MPD in the brain [303;304]. Additionally, intracerebroventricular administration of these two substances produces only small or no increases in behaviors, in contrast to intracerebroventricular administration of a comparable amount of MPD [303;305]. Therefore, we tested only levels of MPD, not levels of ritalinic acid and para-hydroxymethylphenidate, in brain and plasma.

Highlights of Methods

A schematic (**Fig. 19**) summarizes the behavioral procedure for Experiment 3 (for detailed information of subjects and assay procedure, please see **Supplementary Methods**). Following the elevated plus-maze test, that is, 35 min after oral administration of MPD at 3 mg/kg or vehicle, the

MPD-treated rats were decapitated. Brain and plasma of trunk blood was collected. Samples were stored at -80 °C.

Figure 19. Design of Experiment 3.



Data Analysis

Three mixed linear models were constructed with dependent variables of drug level in brain, in plasma, and brain/plasma ratio, separately. Fixed factor was age. Litter and baseline activity level were controlled as a random factor and a linear covariate, respectively. Satterthwaite adjustments were applied to denominator degrees of freedom.

In addition, general mixed linear models were used to examine the correlation of the 4 measures of the elevated plus-maze test with plasma, brain levels of MPD, and the brain/plasma ratio, respectively. Age was a fixed factor; litter was a random factor.

Results

As illustrated in **Fig. 20**, brain and plasma levels of MPD changed significantly across age ($F[2,18.4]=14.52$, $p<0.0012$; $F[2,17]=8.41$, $p=0.0029$), but the brain/plasma ratio did not differ among age groups. Brain levels of MPD in Age 1 were higher than those of both Age 2 ($p<0.001$) and Age 3 ($p<0.001$). Brain levels of MPD in Age 2 were marginally higher than in Age 3 (unadjusted $p=0.032$, adjusted p=0.079). Plasma MPD level in Age 1 was significantly higher than in Age 2 (p=0.044) and Age 3 (p=0.002); the plasma MPD level of Age 2 was significantly higher than in Age 3 (p=0.031).

Correlation of Brain, Plasma, and Brain/Plasma Ratio of MPD with Behaviors on the Elevated Plus-Maze

With behavioral measures on the elevated plus-maze as dependent variables, we detected no significant effects of individual brain levels, plasma levels, or brain/plasma ratio of MPD. No significant interaction was detected between age and individual brain or plasma levels or brain/plasma ratio of MPD. For the completion of data, a summary of results of Pearson correlation analyses is shown in **Supplementary Table 1**.

Summary

In rats, oral MPD at 3 mg/kg on PND 24 produced high levels of MPD in the brain and the plasma, but both levels decreased by PND 30 and again by PND 37. Individual brain and plasma levels and the brain/plasma ratio rarely correlated with behavioral measures on the elevated plus-maze.

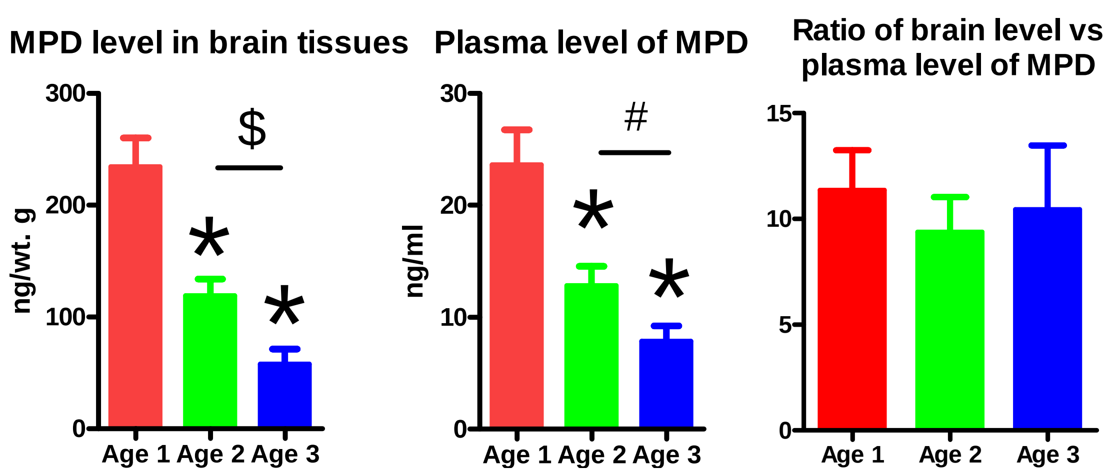


Figure 20. Age effect on the metabolism of MPD at 3 kg/mg. (N=7-8/age group) Mean ± SEM of brain and plasma levels of MPD shown for age group. * Significant difference from Age 1 (p<0.001); $ Marginally significant difference between Age 2 and Age 3 (adjusted p=0.079); # Significant difference between Age 2 and Age 3 (p=0.031)

Experiment 4. Individual baseline locomotor activity in a novel environment as a predictor of behavioral responses to MPD in three age ranges of pre- and peri-adolescence.

Hypothesis: Individual baseline levels of locomotor activity (response to novelty) will predict individual behavioral response to oral MPD, including cognitive performance, anxiety-like behaviors, and locomotor activity.

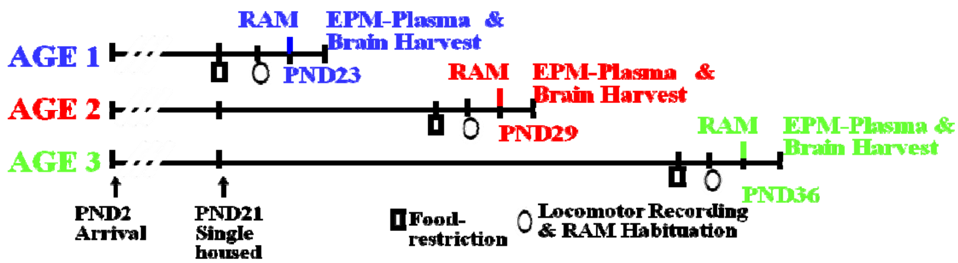
Rationale: One focus of ADHD research is identifying predictors of treatment (e.g., MPD) outcome [306-308]. Recently, evidence emerged indicating that cognitive improvement by MPD correlates with individual baseline performance, although the reported directions of correlations were contradictory [135;309].

We found that individual differences in locomotor response to MPD positively correlated with locomotor response to novelty (**Preliminary Experiment 2, Fig. 7**). The attention task also revealed seemingly individual differences in response to MPD (**Preliminary Experiment 2, Fig. 6**). Individual differences in response to MPD are well-documented in humans [for a review, see 310]. Behavioral responses to MPD in other domains possibly correlate with response to novelty. But no publication has investigated this possibility in pre- and peri-adolescent animals. Understanding of individual differences in cognitive and other responses to MPD, such as anxiety-like behaviors, will help interpret the results of related studies and possibly provide information for predicting the treatment outcome of MPD in clinical settings.

Highlights of Methods

Fig. 21 summarizes the timeline of Experiment 4 (for detailed information of subjects, apparatus and behavioral procedure, please see **Supplementary Methods**). Locomotor recording is indicated by an **O** symbol. In all experiments, one day before radial arm maze testing, rats were individually placed into Digiscan Activity Monitors. Total distance traveled in cm was recorded in 5-min time blocks for a total of 15 min. Recording took place with no drug administered.

Figure 21. Design of Experiment 4.



Data Analysis

Table 5. Measures Analyzed for Correlation with Baseline Activity.	
Experiments	Measures
1	*Corrects to Repeat*
2	*Open Arm Time*, *Percent of Protected Stretch-Attend Postures*, *Entries to Enclosed Arms*, *Grid Crossing*
3	Brain MPD level, plasma MPD level, brain/plasma ratio

Generalized linear models determined the effect of individual baseline activity, and interaction of individual baseline activity and age, treatment, and age and treatment in behavioral measures. For all analyses, fixed factors included treatment, age, and day (if applicable). Individual total distance traveled in the first 15 min in a novel environment was considered a continuous predictor. For all non-adult analyses, litter effect was controlled by introducing it as a random factor. **Table 5** summarizes the behavioral measure analyzed for correlation with individual baseline activity. Separate Pearson correlation analyzed the correlations within age groups and between age groups.

Results

Corrects to Repeat on the Radial Arm Maze

We found no significant effect of baseline activity or interaction of baseline activity and treatment, baseline activity and age, or baseline activity, treatment and age. Individual baseline activity did not predict individual performance on radial arm maze. For the completion of data, correlation coefficients for baseline activity and measures on the radial arm maze are summarized in **Supplementary Table 2**.

Measures on the Elevated Plus-Maze

We found only significant effects of individual baseline activity in *Entries to Enclosed Arms* ($F[1, 23.2]=33.49$, $p<0.001$) and *Grid Crossing* ($F[1, 38.2]=14.15$, $p<0.001$). Individual baseline activity predicted *Entries to Enclosed Arms* and *Grid Crossing*. However, there was no significant interaction among treatment, age, and/or baseline activity in all measures. For the completion of data, *Entries to Enclosed Arms* and *Grid Crossing* on the elevated plus-maze are plotted against individual baseline activity in **Supplementary Figure 9A** and **9B**, respectively.

Summary

Individual response to novelty was only correlated with overall locomotor activity on the elevated plus-maze (*Entries to Enclosed Arms* and *Grid Crossing*), but not with measures of anxiety on the elevated plus-maze, nor measure of performance on the radial arm maze.

Discussion

Several questions about MPD await answers. For one, the mechanisms underlying the therapeutic effects of MPD are under intensive investigation in human and animal studies. Existing behavioral and imaging data of humans demonstrate the importance of several structures such as the PFC, striatum, and cerebellum, as well as the limbic system. Meanwhile, very few studies have investigated the effects of MPD on emotion.

Animal studies have contributed to the understanding of the actions of MPD. However, MPD is primarily prescribed for children and adolescents, and few studies have investigated its therapeutic efficacy specifically in pre- and peri-adolescent animals. As reviewed in the Introduction, the brain undergoes substantial changes throughout childhood and adolescence. Thus, animal studies on the actions of clinically-relevant doses of MPD in corresponding age groups are not only needed, but indispensable.

Since MPD has been predominantly reported to improve cognition, reduce hyperactivity, and possibly affect emotion-related function in both individuals with ADHD and in healthy individuals [133-137;195;311;312], the present study investigated the actions of clinically-relevant doses of MPD in pre- and peri-adolescent rats on cognitive performance, anxiety, and activity. We also examined the role of individual differences in behavioral responses to the drug, to help provide a clearer view of the behavioral data and possibly contribute to finding a predictor of treatment response in clinical conditions.

Overview of Results

Based on our preliminary study, we predicted that (1) the effects of oral MPD at clinically-relevant doses on performance on the radial arm maze are age-dependent; (2) oral MPD alters anxiety-related behaviors and locomotor activity in an age-dependent manner, both of which contribute to the effects of MPD on performance on the radial arm maze; and (3) individual responses to novelty (i.e., baseline locomotor activity in a novel environment) predict individual behavioral responses to oral MPD, including cognitive performance, anxiety-like behaviors, and locomotor activity. Findings of the present study supported our first and second hypotheses, but contradicted the third hypothesis.

The main finding of this study is that oral MPD affects performance on the radial arm maze, anxiety-related behaviors, and locomotor activity. These behavioral effects demonstrated differential profiles across ages during pre- and peri-adolescence. That is, oral MPD at 3 and 4 mg/kg transiently improved accuracy on the radial arm maze during PND 36-37, predominantly through facilitating the rat's movement on the radial arm maze In addition, oral MPD at 3 mg/kg prominently and consistently produced an anxiolytic-like effect in the elevated plus-maze on rats at ages PND 24, 30, and 37; this treatment increased locomotor activity on PND 24 and (less so) on PND 37. Further, brain and plasma levels of MPD produced by the same acute dose decreased across ages. However, this decrease does not fully explain the age-dependent profile of the three behavioral effects. The separability of drug effects on behaviors at each age point suggests that the effects of MPD are manifested through relatively independent pathways.

We also report here that individual differences in locomotor response to novelty only predicted overall locomotor activity but did not predict the magnitude of MPD-induced changes in locomotor activity on the elevated plus-maze, anxiety-related behaviors, or accuracy of performance on the radial arm maze. This, too, is consistent with the notion of multiple, independent effects of MPD. However, further investigation may be needed to determine whether and how individual differences in a given domain of function predict response to MPD in the same domain and under the same testing paradigm.

Radial Arm Maze

When controlled for individual bodyweight change (see **Supp. Method** for details) and baseline locomotor activity, oral MPD at 3 and 4 mg/kg increased *Corrects to Repeat*, the main measure of accuracy in Age 3 (PND 36-37, but not PND 38-41). In contrast, all doses had no significant effect in this measure for Age 1 pups (PND 23-28), Age 2 pups (PND 29-34), or Adults (PND 81-86) when analyzing all rats.

Interestingly, the improvement in accuracy during PND 36-37 in the MPD-treated group disappeared after removing rats that did not commit an error due to low number of total entries on the radial arm maze (**Fig. 11**). Too low a number of entries tends to prevent the occurrence of revisiting error and therefore discredits the accuracy measures (e.g., *Corrects to Repeat* and *Errors in 8 Entries*). In this condition, low scores in *Corrects to Repeat* and *Errors in 8 Entries* did not represent the level of spatial learning and working memory and therefore should be excluded.

The disappearance of treatment effect in Age 3 after data refinement logically suggests that oral MPD induced an apparent improvement of accuracy by facilitating the rats to move on the radial arm maze, when, in contrast, controls were not readily exploring the maze and not displaying meaningful accuracy scores. However, data from measures of locomotion and efficiency indicated otherwise. Although oral MPD at 4 mg/kg significantly increased total number of entries rats made in a trial across age and trials (**Supp. Fig. 1B**), in Age 3 all treatment groups made a comparable number of entries in the first two trials (**Supp. Fig. 18C)**, during which oral MPD at 3 and 4 mg/kg increased scores of *Corrects to Repeat*. Additionally, oral MPD did not significantly affect all measures of efficiency across trials and ages (**Supp. Fig. 3-7**). Therefore, oral MPD at 3 and 4 mg/kg improved accuracy on the radial arm maze, possibly by enhancing functions other than locomotion.

Since a small sample size in control and treated groups also could diminish significance in statistical analyses (**Fig. 12**), we conducted additional analyses on the refined dataset of *Modified Corrects to Repeat* in Age 3. To detect possible differences in the levels of movement in the rats that remained in the refined dataset, *Total Entries* for these rats (*Modified Total Entries*) were also generated and analyzed. Kruskal-Wallis one-way analyses were applied since *Corrects to Repeat* and *Total Entries* were not normally distributed. One compared controls to rats treated with oral MPD at 3 mg/kg; the other compared controls to rats treated with oral MPD at 4 mg/kg. Although the Kruskal-Wallis test is non-parametric and less powerful than parametric analyses, the results provide a clearer view of the data.

Modified Corrects to Repeat together with *Modified Total Entries* indicated that, only in Trial 1, (PND 36) oral MPD at 3 mg/kg improved accuracy without affecting levels of movement on the radial arm maze. In rats that entered arms and committed errors in Trial 1 (PND 36), oral MPD at 3 mg/kg significantly altered the distribution of *Modified Corrects to Repeat* compared to that of the controls; the group median of rats treated with oral MPD at 3 mg/kg was higher than that of controls (**Supp. Fig. 8A**). On the other hand, *Modified Total Entries* of controls and rats treated with oral MPD at 3 mg/kg were comparable in Trial 1 (PND 36) (**Supp. Fig. 8B**).

In contrast, oral MPD at 3 mg/kg in Trial 2 (PND 37) and 4 mg/kg in both trials did not alter *Modified Corrects to Repeat* and *Modified Total Entries* compared to the controls (**Supp. Fig. 8**). In rats that entered arms and committed errors on Trial 2 (PND 37), the distributions and medians of *Corrects to Repeat* and *Total Entries* were comparable between controls and rats treated with oral MPD at 3 mg/kg. Similarly, in the refined dataset, rats treated with oral MPD at 4 mg/kg performed and moved at a comparable level compared to controls in both Trials 1 and 2. Therefore, oral MPD at 3 mg/kg in Trial 2 and 4 mg/kg in both trials did not affect accuracy when comparing between the controls and treated rats that moved readily at comparable levels. It is thus reasonable to infer that in the raw dataset containing all rats regardless of levels of movement, oral MPD at 3 mg/kg in Trial 2 and 4 mg/kg in Trials 1 and 2 increased *Corrects to Repeat* by facilitating movement of rats that were not readily exploring the radial arm maze.

Another measure of accuracy on the radial arm maze is *Errors in 8 Entries.* Oral MPD at all doses did not significantly affect the number of errors rats made in the first 8 entries (**Fig. 12**). However, *Errors in 8 Entries* is less sensitive than *Corrects to Repeat* in measuring accuracy, since one error occurring early in a trial represents a lower level of accuracy than one error occurring late in a trial. Further, *Errors in 8 Entries* is heavily influenced by movement on the radial arm maze. Low number of total entries naturally reduces the probability for errors to occur. For example, adult rats showed a mean lower than 0.5 for errors in the first 8 entries in the first several trials (**Fig. 12A**). This resulted because adult rats rarely entered any arms in the radial arm maze in the first several trials (**Fig. 18C**). As training proceeded, adults gradually entered more arms and committed revisiting errors, thus *Errors in 8 Entries* increased in later days. Therefore, *Corrects to Repeat* remains the best measure of accuracy in the radial arm maze task.

Hypothetically, a rat relying on a biased response strategy could demonstrate a high level of accuracy without any memory on the radial arm maze. For example, always skipping 2 arms to the left will generate a *Corrects to Repeat* score as high as 8. Oral MPD at 3 and 4 mg/kg in Age 3 could improve accuracy on the radial arm maze by driving the rats to adapt a biased response strategy, without affecting any domain of cognitive function. Since *Corrects to Repeat* was determined within the first 8 entries, strategy in the first 8 entries provides more precise information for interpreting accuracy data. *Strategy in 8 Entries* revealed no significant difference among treatment groups across all ages (**Fig. 17**). Oral MPD at 3 and 4 mg/kg did not affect strategy within the first 8 entries on the radial arm maze in all trials across ages (**Fig. 17**). Therefore, in Age 3, rats treated with oral MPD at 3 mg/kg performed at higher levels on the radial arm maze compared to controls despite using similar behavioral strategies.

When considering strategy within full trials, rats treated with oral MPD at 4 mg/kg—but not other doses—obtained significantly lower strategy scores compared to controls. It is likely that rats treated with oral MPD at 4 mg/kg displayed more random strategies simply by making more entries compared to controls. A large number of entries increases the probability of occurrence of each type of arm choice and therefore "dilutes" the pattern of arm choices. Indeed, only oral MPD at 4 mg/kg increased *Total Entries* (**Supp. Fig. 1**). This pattern mirrored the main treatment effect in *Strategy Score* in full trials.

Notably, effects of oral MPD at 3 and 4 mg/kg on the radial arm maze were found only in the first 2 trials in Age 3, indicating either that rats rapidly developed a tolerance for the drug or that previous experience on the maze improved acquisition in control rats as much as did oral MPD in treated rats. To the best of our knowledge the latter explanation is supported by the literature. In humans, experience with a task has been reported to improve cognitive performance following placebo to a similar extent as does MPD [138]. In contrast, reduced response (tolerance) after repeated exposure to clinically-relevant doses of MPD is rarely reported in either humans or animals. Both humans and animals develop tolerance to psychostimulants such as cocaine and amphetamine [313-316]. However, clinical studies reported that only MPD doses over 60 mg/day induces tolerance in pediatric ADHD patients [317;318]. Note that such doses are high compared to those proven therapeutic by the MTA study (15-50 mg/day [111]). Animal studies typically measure tolerance to MPD using locomotor activity. In rats, only high doses of MPD (10-30 mg/kg) administered by i.p. or s.c. injections have been sporadically reported to induce locomotor tolerance [315] but [255;319]. A handful of animal studies investigated the effects of MPD on cognitive tasks, regardless of routes of administration, but none administered the same dose over consecutive days. These studies tested the effects of different doses on performance, and none reported development of tolerance to MPD in the performance of cognitive tasks (e.g., [185;320]).

In summary, oral MPD produces subtle multifaceted effects with age-dependent differences on performance in cognitive tasks during pre- and peri-adolescence. Importantly, these effects appear to span the cognitive, emotional, and motor domains. In Age 3, oral MPD at 3 and 4 mg/kg increased accuracy on the radial arm maze (PND 36-37); no difference was observed at any dose of oral MPD in Ages 1, 2, or Adults. Specifically, for Age 3, oral MPD at 3 mg/kg significantly changed accuracy without affecting strategies in Trial 1 compared to controls that moved on the radial arm maze at comparable levels. In Trial 2, treatment improved *Corrects to Repeat* by facilitating rats' movement on the radial arm maze, when most of the controls were still not readily exploring the maze. On the other hand, oral MPD at 4 mg/kg improved scores of accuracy, likely by solely facilitating the movement of rats when a large portion of vehicle-treated controls were not readily exploring the radial arm maze.

It is also noteworthy that adult rats rarely entered any arms on the radial arm maze during the first several trials (**Fig. 18C**) and therefore did not demonstrate any effect of oral MPD at all doses on performance during this age period. An overall low level of motivation in these adults could certainly have contributed to the low numbers of arm entries during the first several trials. In the present study, adult rats were food-restricted to 90% of free-feeding bodyweight; in contrast, adult rats in most existing studies were food-restricted to 85% of free-feeding bodyweight for performing in appetitive tasks [321;322]. Moreover, a majority of adult rats did not achieve goal weight (90%) during the first 2 days of testing. Therefore, a greater level of food restriction might have motivated the adult rats to perform better on the radial arm maze and reveal the effects of oral MPD.

Elevated Plus-Maze

The elevated plus-maze test also demonstrated multifaceted effects of oral MPD at 3 mg/kg on anxiety-like behaviors and locomotor activity. These two effects varied across age groups (tested on PND 24, 30, or 37).

Oral MPD at 3 mg/kg produced an anxiolytic-like effect regardless of age. It is well-established that *Open Arm Time* increases when anxiety levels decrease on the elevated plus-maze [for a review, see 323]. Oral MPD at 3 mg/kg increased *Open Arm Time* on PND 24, 30, and 37. Percent of *Protected Stretch-Attend Postures* is a measure of anxiety that tends to increase as anxiety levels increase [324;325] and is disassociated from locomotor activity and exploration [325;326]. Oral MPD at 3 mg/kg decreased percent of *Protected Stretch-Attend Postures* in all three age groups. These data are consistent with our preliminary findings that oral MPD at 3 mg/kg on PND 22 produced anxiolytic-like effects on the elevated plus-maze (Fig. 3).

Additionally, oral MPD at 3 mg/kg increased measures of locomotor activity on the elevated plus-maze in an age-dependent manner. *Entries to Enclosed Arms* and *Grid Crossing* measure locomotor activity [323;325]. On PND 24, rats treated with oral MPD at 3 mg/kg entered the enclosed arms more frequently compared to controls (**Fig. 19**). In contrast, *Grid Crossing* was elevated in the MPD-treated group on PND 24 and slightly on PND 37 (**Fig. 20**).

It is unlikely that the anxiolytic-like effect of oral MPD arises from increased locomotor activity in treated rats. The anxiolytic-like effect was present in all three age groups, but both measures of locomotor activity were unaffected in Age 2, and one measure of locomotor activity was unaffected in

Age 3. Further, factor analysis studies have shown that in both adolescent and adult rats, *Open Arm Time* and percent of *Protected Stretch-Attend Postures* solely load on factor 1 (anxiety) whereas *Entries to Enclosed Arms* and *Grid Crossing* solely load on factor 2 (activity) [323;325].

To date only two other groups have examined the effects of acute MPD in anxiety-related paradigms other than conditioned fear [327;328]. Conditioned fear involves learning and memory, abilities strengthened by MPD. Therefore, it is not surprising that MPD enhanced the expression of conditioned fear [329]. In other studies using the elevated plus-maze, one demonstrated in isolation-reared rats (around PND 50) that i.p. injection of MPD at 3 mg/kg 30 min before testing increases time spent in the open arms without affecting total entries on the maze compared to saline-treated controls [327]. The other study reported that i.p. injection of MPD at 3 and 50 mg/kg increases time spent in the open arms, again, without affecting total entries on the maze compared to saline-treated controls [328]. However, MPD at 3, 5 and 50 mg/kg administered via i.p. injection would produce plasma levels that are much higher than the therapeutic range. It is possible that such high doses of MPD do not produce a clinically-relevant effect of the drug. Further study may be needed to clarify the anxiolytic-like effect produced by relatively high doses of MPD in rats.

In summary, oral MPD at 3 mg/kg produced anxiolytic-like effects in all ages and increased locomotor activity in an age-dependent manner during pre- and peri-adolescence. These data supported our hypothesis that oral MPD produces multifaceted effects during peri-adolescence and that the constellation of such effects changes across ages.

Brain and Plasma Levels of MPD

Assessing brain and plasma levels of MPD allowed us to confirm that oral administration of MPD on a small cracker was effective in delivering the drug in all ages during pre- and peri-adolescence. On PND 24 and 30, MPD at 3 mg/kg produced plasma levels that were within the documented therapeutic range of 8-40 ng/ml (raw data: 10-36 ng/ml, 9.10-19.83 ng/ml, respectively) [275]. On PND 37 (**Fig. 21**), plasma levels of MPD bordered on the lower limit of the documented therapeutic range at 8 ng/ml [258]. Despite this lower level, oral MPD at 3 mg/kg altered anxiety-related behaviors and increased locomotor activity on the elevated plus-maze on PND 37.

These data are in line with the developmental decrease in absorption as well as the developmental increase in expression and activity of the enzyme that metabolizes orally-ingested MPD in the liver and blood (carboxylesterase) [258;330;331]. Oral MPD at 3 mg/kg produced brain and plasma levels that were highest in the youngest rats, and both levels decreased significantly in older rats. Similarly, compared to preschool children, school-aged children with ADHD demonstrate less absorption and faster clearance after taking immediate-release MPD [258]. Meanwhile, carboxylesterase displays an increased activity in both human and rat liver until puberty [332;333]. Thus, our finding fits the developmental profile of decreasing absorption and increasing metabolism of MPD.

In summary, oral MPD presented at 3 mg/kg on a small cracker produced brain and plasma concentrations that were either within the therapeutic range or behaviorally effective through pre- and peri-adolescence. In contrast, the variable effects of oral MPD on the radial arm maze, anxiety-related behaviors, and locomotor activity suggest that the circuits mediating these domains of function change in sensitivity to modulation by MPD across ages.

Individual Baseline Locomotor Activity as a Predictor of Treatment Outcome

Significant effect of individual baseline activity levels was found on *Entries to Enclosed Arms* and *Grid Crossing* on the elevated plus-maze. We detected no significant interaction among individual baseline activity levels, treatment, and age in any measure. Locomotor response to novelty predicted overall locomotor activity on the elevated plus-maze regardless of treatment and age (**Supp. Fig. 9**).

This finding differs from our preliminary data indicating that oral MPD at 3 mg/kg increased locomotor activity in a familiar activity monitor more greatly in rats that had higher levels of locomotor response to novelty on PND 23. The procedural differences between the preliminary and the present studies may have contributed to this apparent difference. In the preliminary study, locomotor response to oral MPD was collected immediately after recording of locomotor response to novelty, in a tightly controlled single testing apparatus (activity monitor). In contrast, the present study collected locomotor response to oral MPD one day after recording the locomotor response to novelty and utilized a distinct paradigm (elevated plus-maze). Further, the structural differences between the enclosed activity monitor and the elevated plus-maze may also have influenced the predictability of the locomotor response to novelty for the locomotor response to oral MPD on the elevated plus-maze.

Baseline levels of a given behavior may predict the behavioral response to MPD only in comparable testing paradigms. For example, individual baseline levels of cognitive performance predict performance on the same task after administration of MPD. One reason is that different functional domains may require different levels of DA and NE for optimum performance. Indeed, one human study demonstrated that MPD in children with ADHD impaired performance in a planning task while improving performance in a spatial working memory task [135]. However, no studies have addressed the mechanism underlying the relationship between baseline cognitive levels and after-MPD performance per se, in humans or in animals. Mehta and colleagues [135] suggested that the circuits mediating cognitive performance are in a hypodopaminergic state in children with ADHD, which is normalized by MPD. Further studies on the predictability of cognitive response to MPD in animals may elucidate the mechanism underlying the predictive value of baseline performance in domains of function.

Possible Mechanisms of Ages-dependent Effects on the Behavioral Tasks

Radial Arm Maze

Oral MPD at all doses did not affect accuracy in Ages 1 and 2 (PND 23-28 and PND 29-34) but did in Age 3 (PND 36-37). Procedural factors are the most likely explanation of different response to oral MPD on the radial arm maze across ages.

In contrast to the present study, our published preliminary radial arm maze study demonstrated improvement and impairment of performance during PND 22-24 and PND 32-34 (**Fig. 3**). Two procedural changes in the current experiment compared to the preliminary study may explain the missing effects of oral MPD. First, the current radial arm maze procedure exposed rats to the testing environment one day before testing by imposing a habituation session. This pre-testing experience of task-related stimuli facilitated movement and performance of rats in Ages 1 (PND 23-28) and 2 (PND 29-34), since controls made more entries before committing an error in the first trial (**Fig. 13**, first and second panels) than controls of equivalent ages in the preliminary study (**Fig. 3**). This result supports the idea that previous experience of a task improves performance in the cognitive task as much as MPD treatment does [138] .

Second, rats in Age 2 (PND 29-34) had no previous MPD exposure. In contrast, rats aged PND 29-34 in the preliminary study received a single daily dose of oral MPD at 3 mg/kg from PND 22-28. Thus, previous MPD exposure may have induced hypersensitivity to the drug in the preliminary radial arm maze study. Gray and colleagues reported that injection of 5 mg/kg MPD for 15 days induced 55% greater immunoreactivity for tyrosine hydroxylase and 40% less NET immunoreactivity density immediately after the dosing period [334]. Increased expression of tyrosine hyroxylase could lead to increased synthesis of DA in the brain; therefore, a dose of MPD that was therapeutic when administered in a drug-naïve rat would now inhibit a similar amount but a higher percentage of NET due to the decreased overall NET density. Since NET takes up both NE and DA in the PFC and hippocampus [290;335], a higher percentage of NET inhibited by MPD would induce too high levels of extracellular DA and NE, producing detrimental effects on behaviors, such as the impaired accuracy on cognitive tasks found during PND 32-34 in the preliminary radial arm maze study (**Fig. 3**).

After accounting for procedural factors and non-cognitive effects of oral MPD, 3 mg/kg of MPD still transiently affected accuracy and increased the group median of *Corrects to Repeat* in Age 3. Control and treated rats in this analysis explored the maze at comparable levels (**Supp. Fig. 8**). Two domains of cognitive function and responsible structures are potential candidates. Oral MPD could enhance spatial learning and working memory, which are dependent on hippocampal function [for a review, see 287]. Lesions of the hippocampus interfere with both acquisition and performance on the non-delayed win-shift radial arm maze [276;336]. Although the PFC is also associated with spatial working memory and win-shift paradigms, animal studies have shown that PFC lesions do not affect free-forging tasks but only tasks containing a delay over 30 seconds, during which the animal is confined or removed from the testing apparatus [336;337].

Both the NE and DA systems modulate hippocampal function. Methylphenidate enhances both long-term potentiation and long-term depression in hippocampal slices in a β-adrenoreceptor-dependent manner [289]. Meanwhile, blockade of DAT and NET in hippocampal slice preparations increases extracellular levels of DA [290]. *In vivo* infusion of D2 agonist quinpirole into the hippocampus dose-dependently improves choice accuracy, while the D2 antagonist raclopride dose-dependently impairs choice accuracy [288]. On the other hand, systemic administration of non-specific A2AR agonist Clonidine does not affect performance on the radial arm maze [338]. D1 agonist and antagonist are also reported not to affect radial arm maze performance [288;292]. Taken together, oral MPD at 3 mg/kg could increase extracellular levels of NE and DA in the hippocampus and enhance plasticity via activation of the β-noradrenoreceptor and D2 receptor.

The role of hippocampus in the actions of MPD requires further clarification. Only a few studies on adult ADHD, but not pediatric ADHD, reported abnormalities of hippocampus and differential response to MPD in hippocampus between patients and typical individuals [339;340]. Animal studies only investigated rats during late adolescence (around PND 48), revealing changes in neurotransmitters in hippocampus induced by clinically-relevant doses of MPD [187]. Similarly, in the present study, the effects of oral MPD were absent in pre-adolescent rats but transiently present in the oldest peri-adolescent group. The role of hippocampus may gradually emerge in the therapeutic actions of MPD in adolescence.

Additionally, oral MPD may facilitate procedural learning, since the drug effect was only present at the beginning of the ascending phase of the learning curve (**Figs. 13 and 14**). As reviewed in the introduction, abnormalities are consistently reported in both the striatum and the cerebellum in ADHD [reviewed in 53;77;78;79;80]. Interestingly, both the striatum and the cerebellum have been implicated in non-motor procedural learning in acquisition of cognitive tasks ([reviewed in 341;342]). For example, Poldrack and colleagues [343] showed in humans that the right caudate nucleus is activated during learning of an implicit cognitive task [344], using a control task that requires only perceptual and motor response. Additionally, striatum lesions in rats interrupt performance in the water maze after 4 training trials but not after 12 training trials [345]. Cerebellum has been shown to be activated after a procedural version of water maze, compared to a spatial version of the task [346].

Although ADHD patients have demonstrated abnormalities in both striatum and cerebellum (reviewed in [347]) and differential activation of striatum to MPD (reviewed in [197]), non-motor procedural learning has been studied little in ADHD. Animal studies have not examined the possible effects of MPD on procedural learning. The few studies that did investigate procedural learning in ADHD reported controversial results [348;349]. The missing piece of research is partially due to difficulty in finding and interpreting measures of non-motor implicit learning. Since procedural learning is ubiquitous in human life, further work may be needed to clarify the role of non-motor procedural learning in ADHD and the actions of MPD.

Elevated Plus-Maze

In the elevated plus-maze test, oral MPD at 3 mg/kg produced an anxiolytic-like effect at all three ages (PND 24, 30, and 37). Accounting for the developmentally-decreasing absorption and/or increasing metabolism of MPD (**Fig. 20**), the constant presence of an anxiolytic-like effect from a constant dose of oral MPD may, in fact, indicate that the mediating circuit increases sensitivity to the effects of oral MPD during development.

PFC DA modulation likely plays a role in the anxiolytic-like effects of oral MPD, although hippocampus is well-established to regulate the anxiety/stress-related behaviors. PFC, specifically the medial PFC, serves as a "brake" on the expression of anxiety-related behaviors through amygdalar processes, since lesion of the region or depletion of DA in the area exaggerated anxiety-related behaviors [202;350] . It has been shown that PFC regulates anxiety levels on the elevated plus-maze [351;352]. Mizoguchi and colleagues found that elevated DA transmission in the PFC correlates with reduced anxiety-related behaviors on the elevated plus-maze [353]. Additionally, depletion of DA in the medial PFC in rats reportedly reduces time spend in the open arms on the elevated plus-maze. [202]. In contrast, the hippocampal-septal system seemingly drives the expression of anxiety/stress-related behaviors, since lesion of ventral hippocampus reduced anxiety-like behaviors both on elevated plus-maze and in conditioned fear, without affecting spatial learning [354;355].

The developmental trajectory of PFC is complicated. At least in the rat PFC, development of the DA system seemingly suggests an increase in sensitivity to MPD modulation. Rat PFC shows peak values of DA fiber density and DA concentrations around PND 35 (or between 4 and 6 weeks) [222;227]. Meanwhile, NET, which also takes up DA in the PFC [356-358], presents in the forebrain at higher levels on PND 25 and declines to adult levels in late adolescence [359;360]. Therefore, on PND 24, reaching optimal extracellular levels of DA in the PFC may require a high concentration of MPD that sufficiently inhibits both DAT and NET. In contrast, a lower level of MPD may be sufficient after PND 25 when the level of NET declines and availability of DA increases. On PND 35, an extremely low level of MPD is sufficient to increase extracellular levels of DA since DA availability is at its peak.

Additionally, oral MPD at 3 mg/kg on PND 24 increased locomotor activity when measured by *Entries to Enclosed Arms* and *Grid Crossing*. Treatment on PND 37 increased *Grid Crossing* but not *Entries to Enclosed Arms*. This is consistent with the notion that feedback regulation of DA tone mediated by presynaptic autoreceptors appears after PND 28. Shalaby and colleagues reported that the feedback inhibition of DA synthesis by presynaptic D2 autoreceptor only starts to function after PND 28 and strengthens after PND 35. Systemic injection of apomorphine (a D1/D2 agonist) at a low dose (0.05 mg/kg) increases locomotor activity in rats younger than PND 28 but decreases locomotor activity (measured by quadrant entries) in rats older than PND 28 [361;362]. Only after PND 35 does low-dose apomorphine decrease locomotor activity measured by distance traveled. Interestingly, in Shalaby's studies [361;362], a slightly higher dose (0.1 mg/kg) of apomorphine does not affect locomotor activity on PND 28 and 35. Therefore, a dose that is slightly lower than oral MPD at 3 mg/kg may, in fact, decrease locomotor activity in our rats on PND 30 and 37.

Our results, however, contradict the recent literature indicating that very low doses of stimulants reduce locomotor activity in typical humans and normal animals, especially under arousing conditions (reviewed in [311;363]). Kuczenski and Segal [187] found that 2.5 mg/kg MPD via oral gavage reduced locomotor activity in normal rats on PND 51, with locomotor activity measured using quadrant crossing in the recording box. Dafny et al. conducted the only three studies on adolescent animals (rats at PND 40) that investigated locomotor effects of MPD at low doses [255;319;364]. They showed that i.p. injection of MPD at 0.6 mg/kg did not increase distance traveled in a 2-hour period of recording. In adults, Berridge et al. and Arnsten et al. reported that oral MPD at 2 mg/kg enhanced cognitive performance but did not affect locomotor activity, measured by quadrant entries in the recording box [321;365].

The most parsimonious conclusion from the contrast between the present study and recent literature is that 3 mg/kg of oral MPD is relatively high, especially for animals age PND 24. Indeed, rats at PND 24 may be more sensitive to changes in the availability of DAT levels and/or extracellular DA levels compared to the adult rats. For one, the inhibitory modulation of the presynaptic autoreceptors at PND 24 may be non-functional [361;366] or less sensitive [279]. Therefore low doses of oral MPD may not be able to suppress locomotor activity in rats before PND 28. However, as discussed earlier, a dose of oral MPD that is lower than 3 mg/kg may suppress locomotor activity in rats after PND 28 when presynaptic autoreceptors exert inhibitory modulation of DA synthesis.

The nucleus accumbens likely mediates the locomotor effect of oral MPD at low doses. This structure has been widely implicated in locomotor response to psychostimulants (reviewed in [367]). Low doses of DA infused into the nucleus accumbens have been reported to restore spontaneous locomotor activity that was suppressed by activation of amygdala , which is typically induced by stressful stimuli [368]. The developmental profile of the inhibitory autoreceptor regulation of DA transmission in nucleus accumbens also supports its role in the present locomotor effects of oral MPD at different ages. Anderson and colleagues [279] demonstrated that the same dose of D2/D3 agonist (7-OH-DPAT) inhibited DA synthesis in the rat nucleus accumbens at PND 30 but not at PND 20. These data support the notion that the autoreceptors are highly functional after PND 28 but not in rats of younger ages.

Seeman and Madras [194;195] speculated that presynaptic D2 autoreceptors in subcortical areas are responsible for the “anti-hyperactivity” effects of stimulants in ADHD. To our knowledge, functionality of presynaptic D2 autoreceptors has only been studied in peri-adolescent humans (12-15 years) [369;370] but not younger children, partially due to ethical issues. If D2 autoreceptors are indeed not fully functional in children with ADHD, MPD may exert its effect through other mechanisms, for example, by strengthening inhibitory control of PFC over subcortical areas, as hypothesized by Arnsten and colleagues (reviewed in [55]).

Summary

In this study we report multifaceted effects of low-dose oral MPD on cognitive performance, anxiety-related behaviors, and locomotor activity. The constellation of these effects changes across ages. Further, the developmental trajectories of the drug effects reflect the sensitivity to MPD and functional maturation of circuits mediating each domain of effect, which provides information for studying these circuits. The hippocampus may begin participating the cognition-enhancing effect of oral MPD as animals enter adolescence. Oral MPD may also affect procedural learning, but this notion requires further investigation. The PFC likely mediates the anxiolytic-like effect of oral MPD at 3 mg/kg, but the question remains unanswered regarding which structure mediates its locomotor effect.

Comparing the Cognition-Enhancing Effects of MPD in Animals and Humans

The present study, together with the published preliminary study, represents, to our knowledge, the first animal studies investigating the effects of orally-administered, clinically-relevant doses of MPD on performance in cognitive tasks, anxiety-related behaviors, and locomotor activity during pre- and peri-adolescence.

Combining the present study and our previous publications gives rise to the inference that oral MPD at low doses may lead to improved performance on cognitive tasks through non-cognitive effects. Previously, we demonstrated that oral MPD at 3 mg/kg improved accuracy scores on the radial arm maze in rats aged PND 22-24, as well as producing an anxiolytic-like effect. In the present study, a session of habituation abolished the performance-enhancing effect of oral MPD at 3 mg/kg on the radial arm maze during an equivalent age (PND 23-24) although we replicated the anxiolytic-like effect of the drug. Therefore, the improvement found in our previous study was likely due primarily to an MPD-mediated anxiolytic-like effect in un-habituated rats.

Other groups have demonstrated a cognition-enhancing effect of oral MPD at low doses in adult rats and monkeys. Berridge and colleagues [321;365] reported that oral MPD at 2 mg/kg in adult rats improved accuracy in performing a delayed non-match to sample spatial working memory task during the light phase of the dark/light cycle. The effect of oral MPD at low doses was not tested during acquisition of the task. Arnsten and colleagues [186] demonstrated that oral MPD at low doses (individual doses varied between 1.0-2.0 mg/kg) improved accuracy of adult rats performing the same task, again during the light phase of the dark/light cycle. Arnsten's group also showed enhancement of performance by oral MPD at individually adjusted doses (0.1 mg/kg-2.0 mg/kg) in adult monkeys performing an equivalent non-match to sample spatial working memory task [185].

In contrast to the aforementioned studies, the present work administered oral MPD and tested the rats during the dark (active) phase of the rat's circadian cycle. Dosing and testing during the inactive phase of a rat's circadian cycle may augment the beneficial effect of MPD. MPD is well-documented to promote wakefulness in both humans and animals [371]. In one of Berridge's studies on adult rats, i.p. injection of MPD at 0.25 mg/kg produced plasma levels of MPD comparable to the cognition-enhancing dose of oral MPD. Interestingly, i.p. injection of MPD at 0.25 mg/kg also significantly increased time spent awake and decreased time spent in slow-wave sleep [321]. However, rhesus macaques are diurnal animals and benefit from oral MPD in a cognitive task when being tested during the light phase [185]. Therefore, the cognition-enhancing effect of oral MPD in adult animals is likely independent of promoting wakefulness.

Additionally, within-subject design may be mandatory for revealing the cognition-enhancing effect of oral MPD. The present study found only a subtle effect of oral MPD on the accuracy of performance in a cognitive task using between-subject design, which is dominant in animal studies. In contrast, all the aforementioned animal studies used within-subject design, which is widely used in clinical trials. Arnsten and colleagues demonstrated that within each rat the effects of oral MPD varied abruptly across a dose range with 0.5 mg/kg step on performance in their spatial working memory task [186]. In individual monkeys, changes of 0.1 mg/kg in the dose of oral MPD differentiated between enhancing and impairing doses [185]. This is not surprising due to the delicate interplay between the DA and NE systems in the brain, especially in the PFC. Therefore, arbitrarily assigning doses and pooling raw data across animals may "dilute" the effect of oral MPD on cognitive performance. Further, animal studies using within-subject design may provide more information on the actions of oral MPD at clinically-relevant doses.

The effects of oral MPD may be different on performance of a learned task compared to learning a task. The present study tested the effect of oral MPD during the ascending phase of the learning curve. All aforementioned animal studies tested the effect of oral MPD after the subjects had reached asymptote in the respective tasks. In human studies, children with ADHD are also tested on performing tasks. Training developing animals and testing oral MPD effects on performing a cognitive task may be necessary for completing the profile of the effect of oral MPD on cognition in development.

It is also possible that animals, and perhaps children, are less sensitive to the enhancing effect of MPD on higher-order functions such as working memory. The minimal effect of oral MPD on the radial arm maze but prominent effect of the drug on the elevated plus-maze may indicate a predominant effect of oral MPD on low-order cognitive function throughout childhood and adolescence. The literature documents that MPD improves performance in humans in a specific behavioral-function-dependent manner. A meta-analysis of the cognitive effect of immediate-release MPD in children with ADHD reported that 70.6% and 69.7% of studies found positive effects of MPD on performance in attention/vigilance and inhibitory control tasks, respectively, but only 50% of studies using tasks of working memory/divided attention showed beneficial effects of MPD [372]. A typical brain may be even less sensitive to the cognition-enhancing effect of oral MPD. In healthy humans, a 2010 meta-analysis of the effects of MPD [249] reported that only 28% of studies showed improving effects of MPD, combining results from tasks testing for planning, inhibitory control, attention, memory, and working memory. Only one study on the effects of MPD on cognition was found for healthy children (mean age 11.4 years), which reported that MPD improved performance in a memory scanning task and also improved mood [312]. Simultaneously evaluating the acute effects of MPD on a spectrum of cognition and emotional processes may provide more information on how MPD improves behaviors in ADHD.

Of note, the radial arm maze task used in the present study is hippocampus-dependent; the delayed non-match to sample spatial working memory task used in adult studies is PFC-dependent. It is possible that the effects of oral MPD at clinically-relevant doses are not uniform across domains of cognitive function and are therefore task-dependent. An investigation in pre- and peri-adolescent rats using the PFC-dependent spatial working memory task may further clarify the effects of oral MPD on cognition and non-cognitive functions such as anxiety and activity during development.

In summary, the present study found in rats a subtle effect of oral MPD during the learning phase of a hippocampus-dependent task during pre- and peri-adolescence. In contrast, animal studies in adults by others established an effect of oral MPD on performing a PFC-dependent task. Age, task, testing phase, and between-subject designs could all contribute to the differences in the results of the present study on cognition-enhancing effects of oral MPD between animal and human studies.

Conclusions and Implications

The present study is the first to demonstrate that:

1) Oral MPD at performance-enhancing doses produces multifaceted effects, including an anxiolytic-like effect and increased locomotor activity, during pre- and peri-adolescence. Oral MPD at 3mg/kg produces anxiolytic-like effects on the elevated plus-maze on PND 24, 30, and 37, as well as increases locomotor activity on the elevated plus-maze on PND 24 and to a lesser extent on PND 37.

Since oral MPD consistently produces an anxiolytic-like effect across ages, the effect of MPD on emotion may play an important role in improving behaviors and function for individuals with ADHD and healthy humans. In particular, for individuals with ADHD and co-morbid anxiety, the anxiolytic effects of MPD may contribute to the overall improvement of behavior by MPD. Further studies are desirable to clarify the mechanisms of the anxiolytic-like effects of MPD in animals and the potential anxiolytic-like effects of MPD in humans.

2) Oral MPD affects cognition, as shown on a spatial learning and memory task only during the first trial when animals enter adolescence (PND 36). This implies that oral MPD affects, although transiently, hippocampus-dependent working memory and/or procedural learning that depends on striatum and cerebellum. More investigation is needed to clarify the role of the hippocampus in the action of MPD and the role of procedural learning in ADHD.

3) The multifaceted effects of oral MPD on non-cognitive domains of function clearly contribute to the improved performance on a cognitive task, at least in normal animals. Under the conditions of our testing paradigm, the radial arm maze, the effects of MPD on anxiety and locomotor activity are more substantial than the effects on cognition.

Questions and Future Directions

Three issues need immediate investigation:

1. To clarify the effects of oral MPD on PFC during pre- and peri-adolescence, a more time-efficient, re-testable, and prefrontal cortex-specific task is desirable. Delayed spontaneous alternation with food reward may be suitable for this purpose. Within-subject design is preferable to capture the effects of oral MPD.

2. To clarify the mechanisms underlying the anxiolytic-like effect of oral MPD at low doses, firstly, *in vivo* infusion of selective inhibitors of DAT and NET into the PFC and/or amygdala may help isolate the DA and NE contribution to the anxiolytic-like effect of oral MPD under anxiogenic conditions. GBR-12935 [373] and nisoxetine [374], may be utilized as selective DAT inhibitor and selective NET inhibitors, respectively.

3. To clarify the possible effect of oral MPD on procedural learning, comparing the effect of oral MPD on procedural versions of the Morris water maze may be informative. The classic procedural version of the Morris water maze uses a visible (or submerged but cue-marked) platform. Striatal lesions have been shown to disrupt performance in this version of the water maze [345;375]. Another procedural version of the Morris water maze randomly places an invisible platform in the quadrants of the pool, requiring the animal to develop searching strategy. The cerebellum is activated by performing this task [346].

These additional studies could provide more insight into the therapeutic effects of MPD, especially as related to the present studies and as related to the use of MPD in patients with ADHD.

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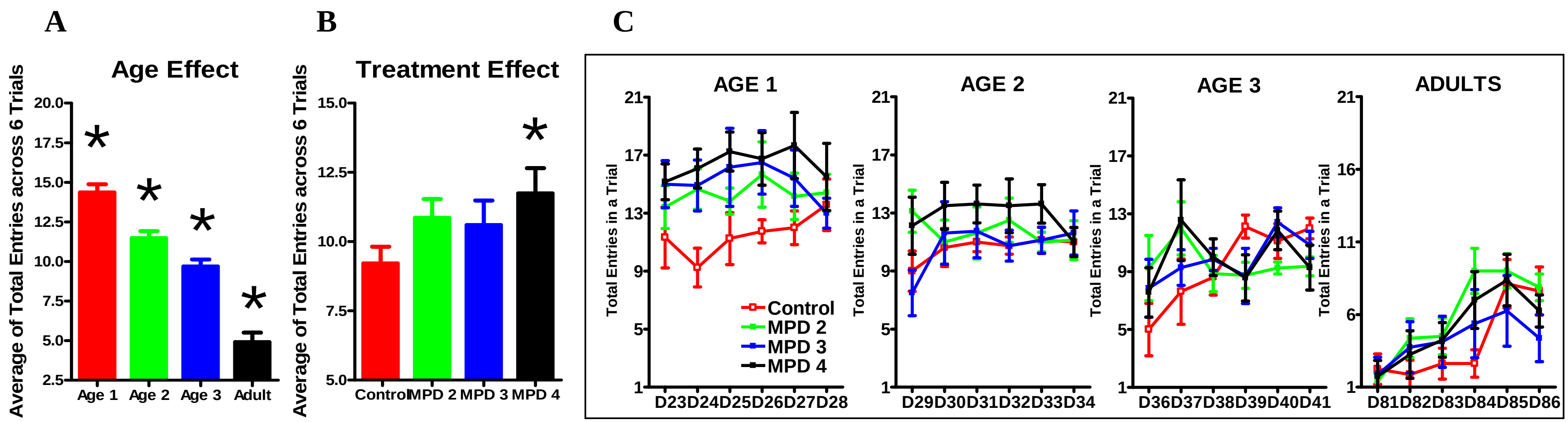
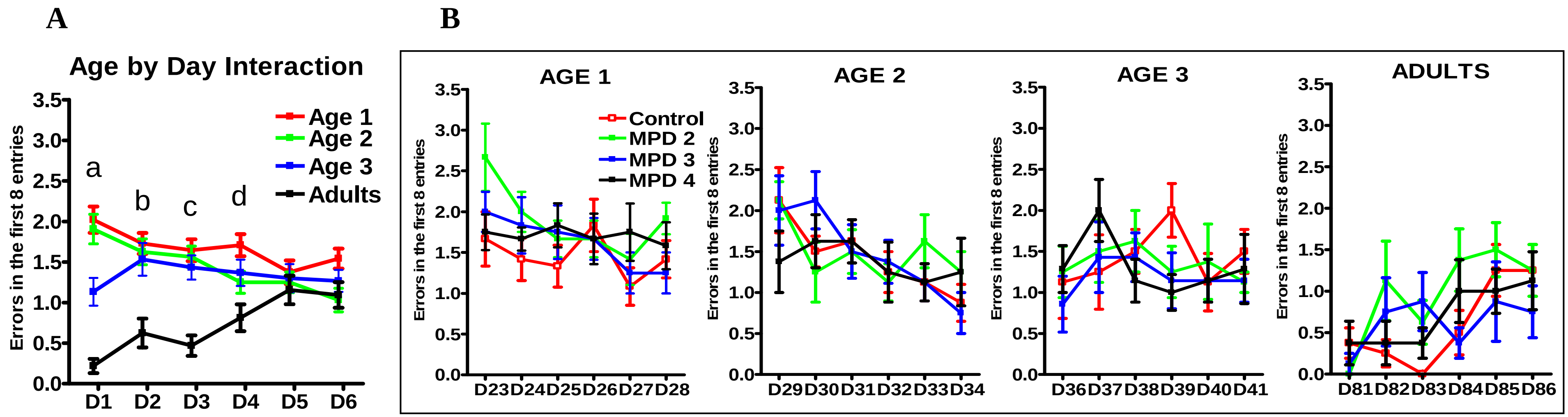
Appendix

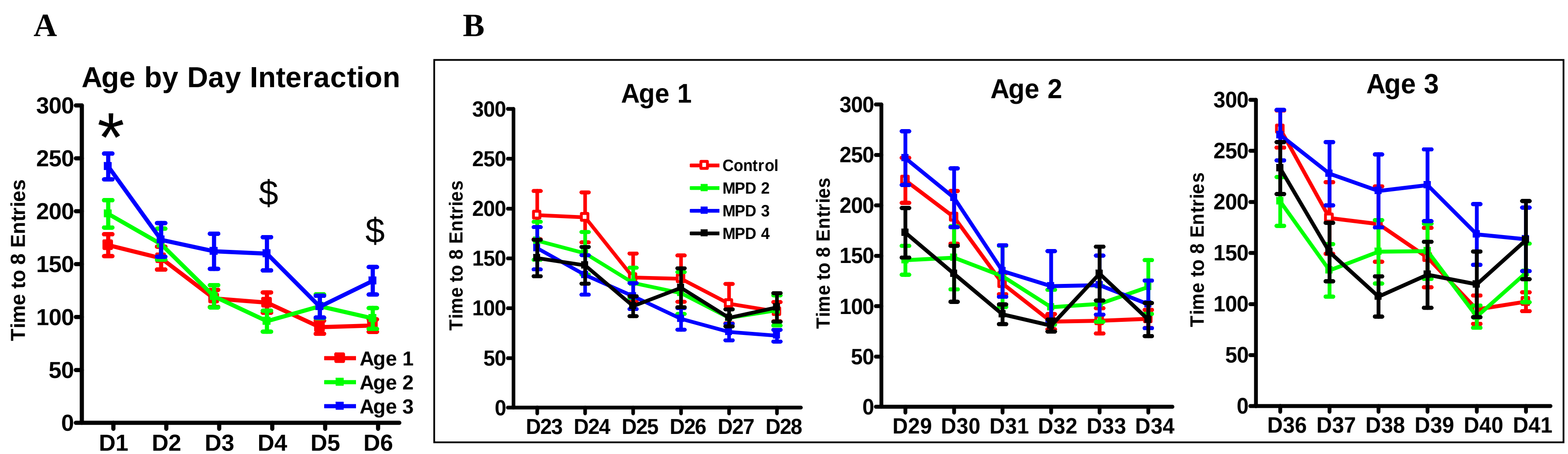
Supplementary Figures



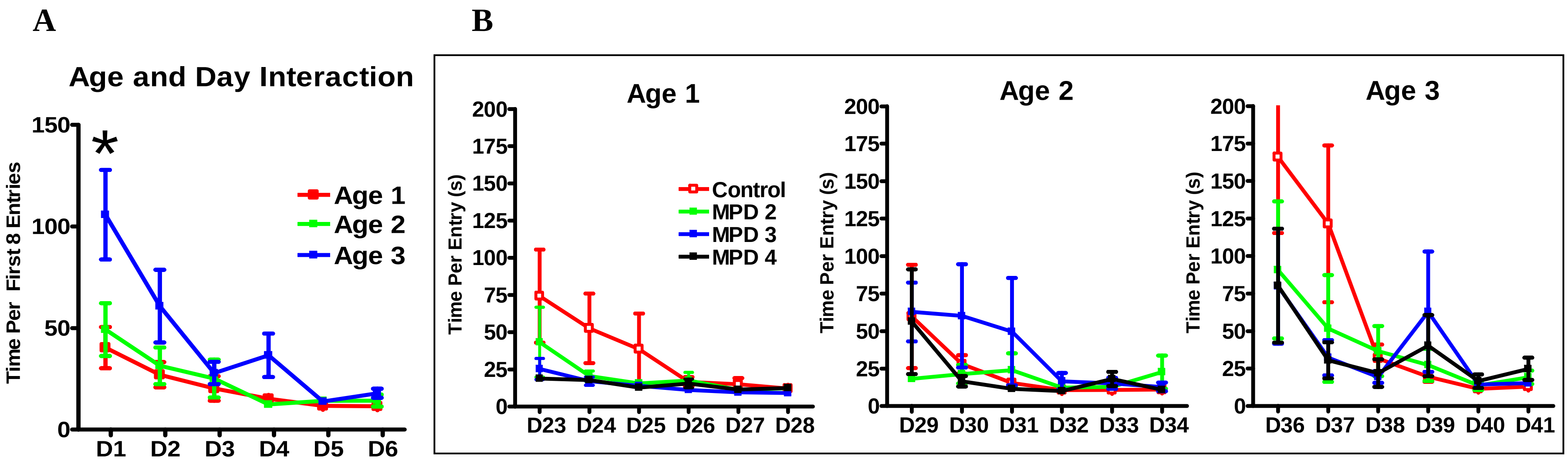
Supplementary Figure 1. Age and treatment effect in *Total Entries* on the radial arm maze. (N=12 or 8 per treatment by age group for Age 1 or all other age groups, respectively) A: Age effect shown by Mean±SEM of *Total Entries* averaged across days for illustration purpose. * Significant difference compared to other age groups (p<0.01). **B:** Treatment effect shown by Mean±SEM of *Total Entries* averaged across trials. * Significance between Dose 4 and the control (p=0.01). **C:** *Total Entries* of treatment by age groups across 6 trials. No significant change across trials.



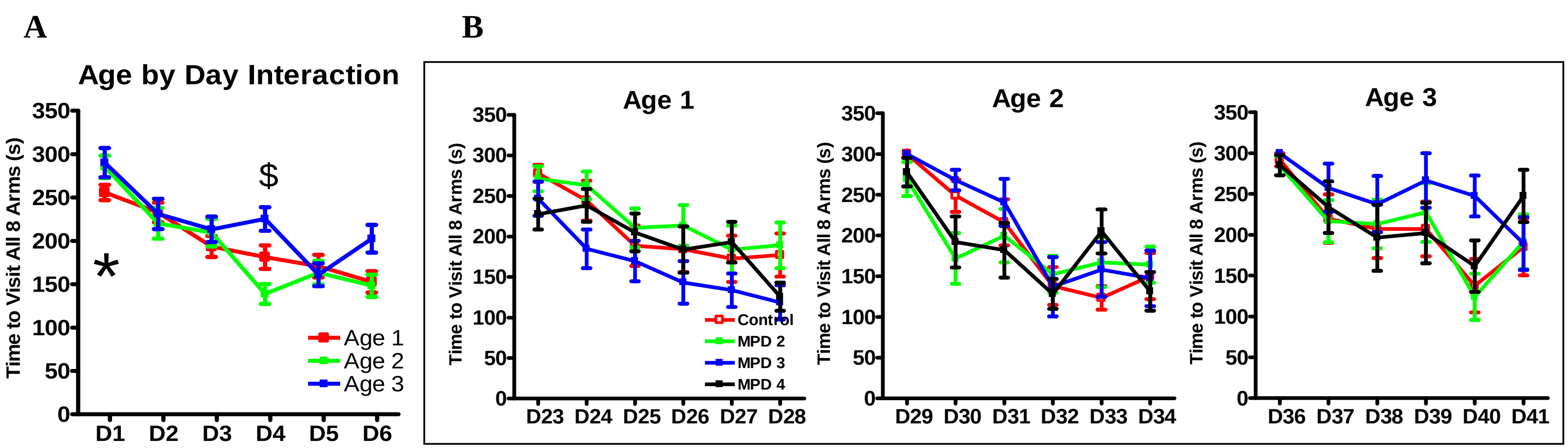
Supplementary Figure 2. Age by day interaction in *Errors in 8 Entries* on the radial arm maze. (N=12 or 8 per treatment by age group for Age 1 or all other age groups, respectively) A: Mean±SEM of *Errors in 8 Entries* shown for age groups across trials. **a** Significance between Ages 1 and 3; Ages 1, 2, and 3, and Adult (p<0.005). **b** Significant difference of Adults compared to Ages 1, 2, and 3. **c** Significant difference of Adults compared to Ages 1, 2, and 3. **d** Significant difference of Adults compared to Age 1. **D**= testing day. **B:** Mean±SEM of *Errors in 8 Entries* of treatment by age groups across 6 trials. No significant change across trials.



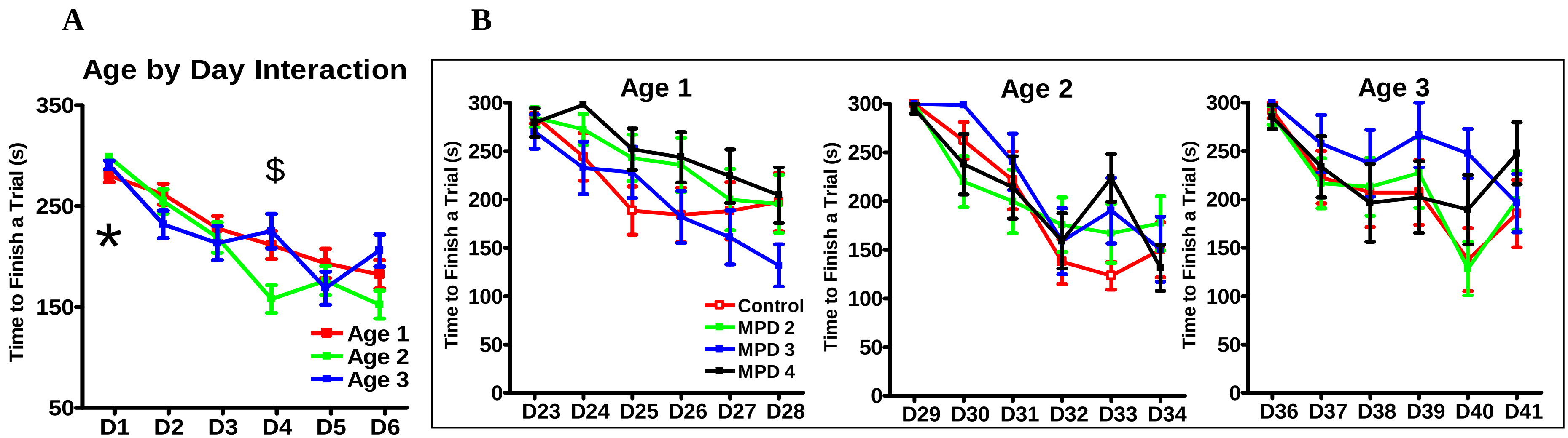
Supplementary Figure 3. Time to 8 Entries. A: Mean±SEM of *Time to 8 Entries* showing age by day interaction (p<0.001). * Significance between Age 1 and Age 2, Age 1 and Age 3, Age 2 and Age 3 (p<0.05). $ Significant difference between Age 3 and Age 1, Age 3 and Age 2 (p<0.05) **B:** Mean±SEM of *Time to 8 Entries* shown for treatment by age groups across 6 trials.



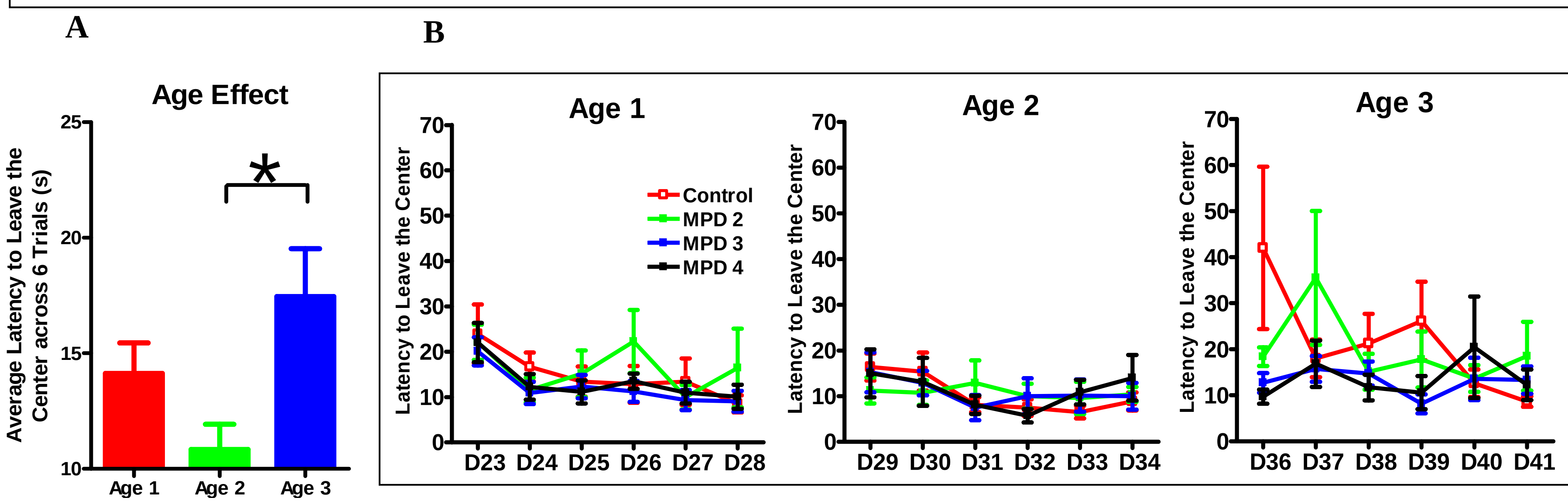
Supplementary Figure 4. Time per Entry in the First 8 Entries A: Mean±SEM of ***Time per Entry in the First 8 Entries*** showing age by day interaction (p<0.01). * Significance between Age 3 and Age 1, Age 3 and Age 2 (p<0.05). **B:** Mean±SEM of ***Time per Entry in the First 8 Entries*** shown for treatment by age groups across 6 trials.



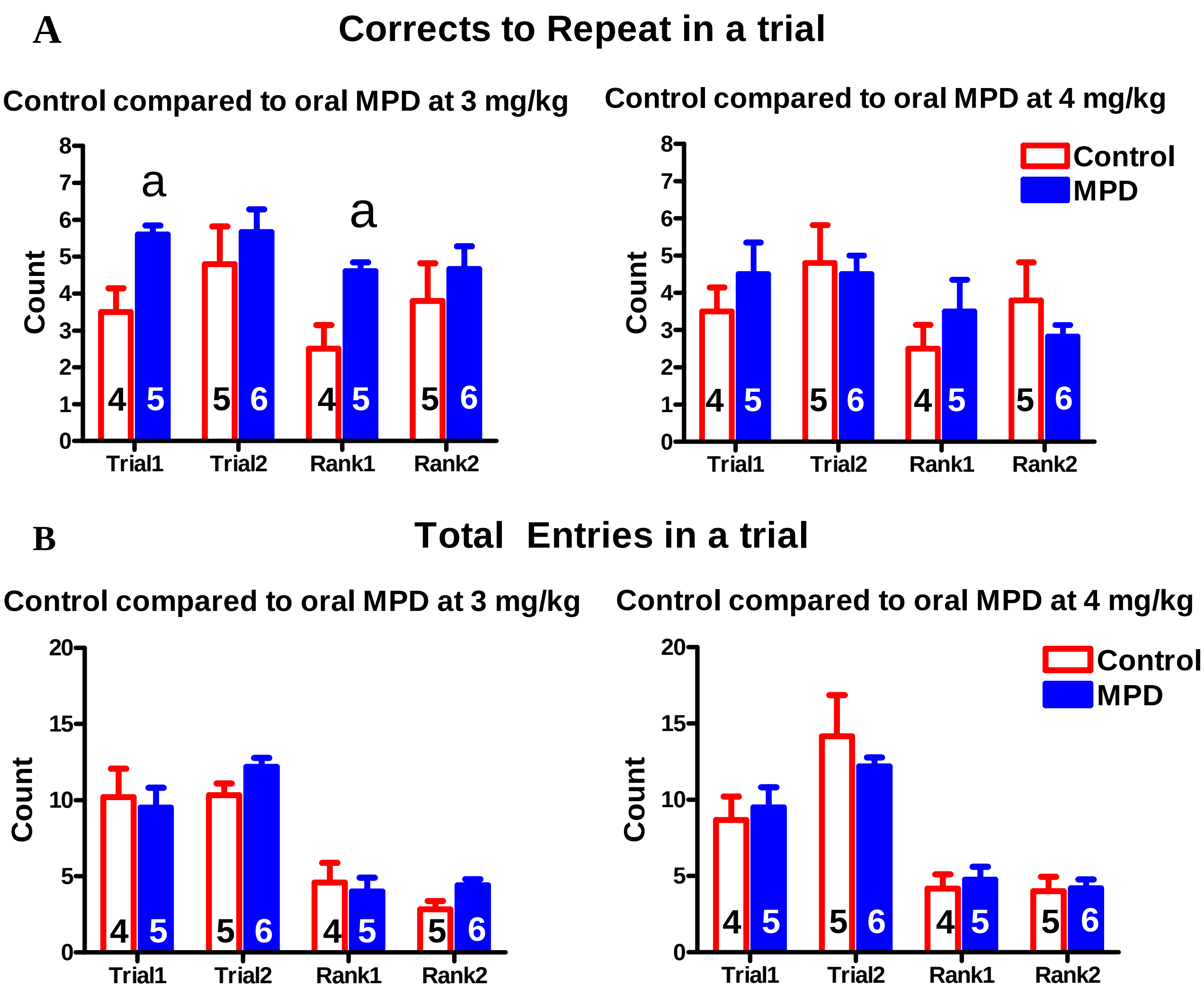
Supplementary Figure 5. Time to 8 Entries A: Mean±SEM of ***Time to 8 Entries*** showing age by day interaction (p<0.01). * Significance between Age 1 and Age 2, Age 1 and Age 3, Age 2 and Age 3 (p<0.05). $ Significant difference between Age 3 and Age 1, Age 3 and Age 2 (p<0.05). **B:** Mean±SEM of ***Time to 8 Entries*** shown for treatment by age groups across 6 trials.



Supplementary Figure 6. Time to Finish a Trial A: Mean±SEM of ***Time to Finish*** in a trial showing age by day interaction (p<0.01). * Significance between Age 1 and Age 2, Age 1 and Age 3, Age 2 and Age 3 (p<0.05). $ Significant difference between Age 2 and Age 1, Age 2 and Age 3 (p<0.05). **B:** Mean±SEM of ***Time to Finish a Trial*** shown for treatment by age groups across 6 trials.



Supplementary Figure 7. Latency to Leave the Center A: Mean±SEM of ***Latency to Leave the Center*** showing main effect of Age (p<0.001). * Significant difference between Age 1 and Age 2, Age 1 and Age 3, Age 2 and Age 3 (p<0.05). **B:** Mean±SEM of ***Latency to Leave the Center*** shown for treatment by age groups across 6 trials.



Supplementary Figure 8. Kruskal-Wallis analysis of raw values and rank values of Modified Corrects to Repeat and Modified Total Entries in Age 3 (N for each group is shown as digit in corresponding bar) A: Mean±SEM of raw values and rank values of *Corrects to Repeat* shown for the controls and the group treated with oral MPD at 4 mg/kg, Trial 1 and Trial 2. **a** Significant difference between the treated group and the controls (p=0.022). **B:** Mean±SEM of raw values and rank values of *Modified Total Entries* shown for the controls and the group treated with oral MPD at 4 mg/kg, Trial 1 and Trial 2.

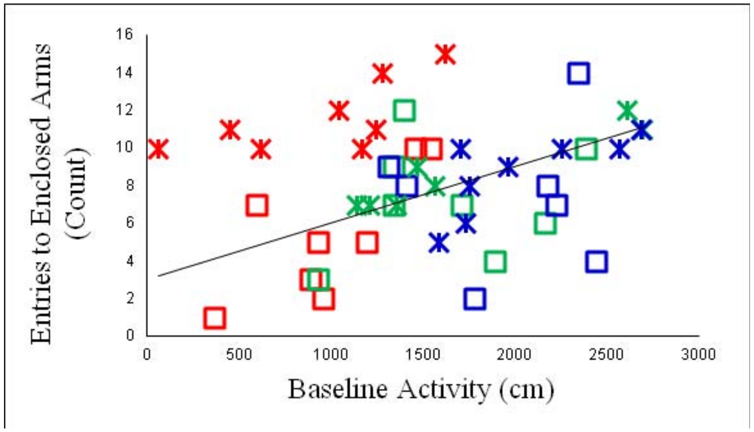
Supplementary Table 1. Correlation of Brain and Plasma Levels of MPD and Brain/Plasma Ratio with Measures from the Elevated Plus-Maze									
	Brain			Plasma			B/P Ratio		
	Age 1	Age 2	Age 3	Age 1	Age 2	Age 3	Age 1	Age 2	Age 3
Entries to EA	**0.873**	0.102	0.164	-0.199	-0.301	-0.545	0.619	0.085	0.649
OA Time	0.638	-0.031	-0.186	0.617	-0.227	-0.217	-0.138	-0.024	-0.336
Grid Crossings	0.643	-0.036	0.068	-0.207	-0.338	0.381	0.590	0.090	-0.244
%PSAP	-0.181	0.361	0.213	*-0.794*	0.190	0.423	0.584	0.122	-0.111

Supplementary Table 1. Correlation of MPD-concentration measures with behavioral measures from the elevated plus-maze. EA: Enclosed Arm; OA: Open Arm; PSAP: Protected Stretch Attend Posture. **Bold** font indicates significant correlation ($p<0.05$); *Italic* font indicates marginal significance.

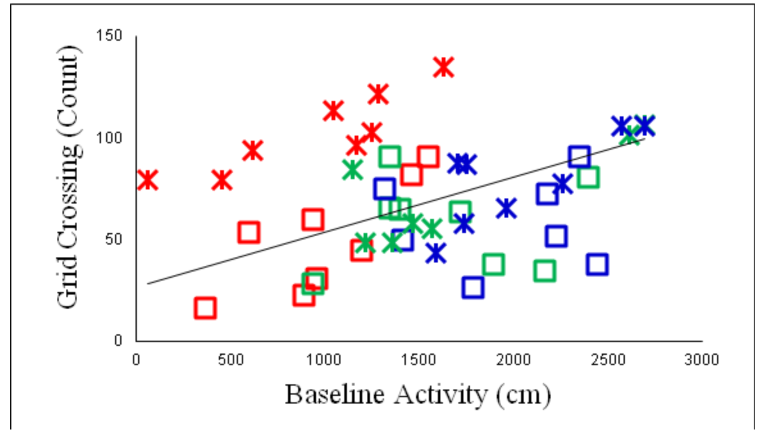
Supplementary Table 2. Correlation of Baseline Activity and Corrects to Repeat from the Radial Arm Maze																
	Age 1 PND 23-24				Age 2 PND 29-30				Age 3 PND 36-37				Adult PND 81-82			
Treat-ment	Ctrl	MPD2	MPD3	MPD4	Ctrl	MPD2	MPD3	MPD4	Ctrl	MPD2	MPD3	MPD4	Ctrl	MPD2	MPD3	MPD4
Trial 1	-0.164	0.369	-0.56	0.114	-0.436	-0.107	0.317	0.66	0.25	0.342	-0.472	0.283	-0.264	-0.381	-0.476	0.7
Trial 2	0.412	-0.123	-0.226	0.051	0.25	0.333	-0.542	0.303	0.28	*0.797*	-0.301	-0.089	-0.181	-0.376	-0.365	0.281

Supplementary Table 2. Correlation of baseline activity and Corrects to Repeat from the radial arm maze. *Italic* font indicates marginal significance.

A



B



Supplementary Figure 9. Correlation of baseline activity and measures of locomotor activity on the elevated plus-maze
A: Regression line showing the relationship between individual baseline activity (response to novelty) and *Entries to Enclosed Arms*. Individual baseline activity predicts individual *Entries to Enclosed Arms* similarly for all age by treatment groups. **B:** Regression line showing the relationship between individual baseline activity (response to novelty) and *Grid Crossing*. Individual baseline activity predicts individual *Grid Crossing* similarly for all age by treatment groups. Red color indicates rats in Age 1; green color indicates rats in Age 2; blue color indicates rats in Age 3. Vehicle-treated controls are illustrated by open squares (□); MPD-treated rats are illustrated by asterisks (*).

Supplementary Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed from PND 2 with a dam and female littermates in a temperature-controlled room in reversed light cycle (lights off from 11:00 am to 11:00 pm). All dams had access to food and water *ad libitum* until PND 21, the day of weaning, when only male rats were single-housed and used for experiments. Food-restriction to 90% of body weight started two days before testing. Target body weight was calculated based on historical age-sex-matched controls. All procedures were be approved by the State Universities of New York Institutional Animal Care and Use Committee.

For the radial arm maze test in adult rats, male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) arrived on PND 60 and were doubly housed in a temperature-controlled room in reversed light cycle (lights off from 11:00 am to 11:00 pm). After two weeks, rats were singly housed for one week. Food deprivation to 90% of body weight started two days before testing. Target body weight was calculated based on individual bodyweight on the day of the beginning of food deprivation.

Apparatus

Locomotor Recording: Digiscan Activity Monitor (model VMRXYZ16; Accuscan Instruments, Columbus, OH) had 48 infrared sensors spaced 2.5 cm apart with 16 sensors along each side to measure horizontal activity. An additional 16 sensors were 10 cm from the floor of the box to measure vertical activity. A transparent Plexiglas box with removable lid (42 L×42 W×30 H cm) confined the animal in the center of the monitor.

Radial Arm Maze: The maze was constructed of gray-painted wood. The center platform was 31.6 cm in diameter while each of the eight arms was 61 cm L×7.8 cm W. A small depression for the bait was located at the far end of each arm. The maze stood 50 cm off the floor in a red-illuminated room with cues placed on each wall. These visuospatial cues remained constant during the entire experiment.

Elevated Plus-Maze: The black Plexiglas plus-maze was elevated 58 cm above the floor. All 4 arms were 51 cm L×11 cm W. Each arm had 3 white-painted grid lines. 42 cm high walls enclosed two opposing arms (enclosed arms, EA). The other two arms (open arms, OA) had only short rails (23 cm L×6 cm H) adjacent to the center start box.

Daily Body Weight Changes

Daily body weights were recorded. Daily weight change was later calculated for individual rats as shown below. Baseline body weight was taken immediately before food-restriction started.

$$\text{Percent of Baseline Body weight} = \frac{\text{Body weight Day X}}{\text{Body weight Day 0}} \text{ x } 100\%$$

Behavioral Procedures

Habituation: In all experiments, rats were habituated to the testing environment and the maze with no treatment for 5 min 1 day before testing. Food rewards were scattered over the entire maze to facilitate habituation.

Locomotor Recording: In all experiments, one day before radial arm maze testing, rats were individually placed into Digiscan Activity Monitors. Total distance traveled in cm was recorded in 5-min time blocks for a total of 15 min. Recording took place with no drug administered.

Radial Arm Maze: On each day of testing, rats received a single dose of MPD of 2, 3, 4 mg/kg or 0.75 ml/kg vehicle (sterile water) on a cracker. 30 min after dosing, each rat was placed on the central platform of the maze with all 8 arms baited with 1 food reward at the end of every arm. The rat was restricted in the center of the maze for 10 seconds to signal the start of the trial. A maximum of 5 min was allotted for each rat to explore the arms in any order or until all 8 baits had been retrieved, whichever came first. Then the rat was returned to its home cage and fed a limited amount of rat chow calculated according to the bodyweight of historical control.

A correct choice was defined as one in which the rat entered and proceeded more than 50% of the way down an arm not previously explored. An error was scored when the rat entered and proceeded more than 50% down an arm it had previously visited.

Elevated Plus-Maze: Rats received one day of radial arm maze testing. On the next day, rats were tested on the elevated plus-maze. On both days, rats received a single dose of MPD at 3 mg/kg or sterile water on a cracker. For testing on the elevated plus-maze, 30 min after dosing, each rat was placed in the start box in the center of the maze, facing the open arm away from the experimenter. After 3 seconds, the start box was removed and the session began. Video recording continued for 5 min. Ethological software (Observer 5.0, Noldus, Netherlands) was used to analyze videotapes for the most well-accepted measures of anxiety-related behavior and locomotor activity on the elevated plus-maze [1-3;4] .

Plasma and Brain Levels of MPD: MPD-treated rats were decapitated immediately following the elevated plus-maze test (35 min after oral administration of MPD at 3 mg/kg). Trunk blood of each rat was collected in a 10 ml heparin-containing tube (Fisher Scientific, Pittsburgh, PA) on ice and mixed 3–4 times. After centrifugation at 4000 rpm for 10 min, plasma was aspirated using a transfer pipette. Brain was removed from the skull and snap-frozen in methylbutane at -20 °C. Decapitation, blood collection, and brain removal were completed within 10 min. Plasma isolation was completed within 20 min. Samples were stored at -80 °C.

Plasma Samples: Plasma samples were shipped on dry ice to the National Center of Toxicology Research, AR. The method used for analyzing plasma levels of MPD was as described by Manjanatha et al. [5]. Briefly, plasma samples underwent solid phase extraction using Spec C-18 96-well plates (15 mg, Varian, Palo Alto, CA). Before sample loading, individual wells were washed once with 1 ml of 2% formic acid in methanol, and then twice with 1 ml of 0.1% aqueous formic acid. After loading samples (0.91 ml), wells were washed once with 1 ml of 5% methanol in 0.1% formic acid (v/v). Elution was performed three times with 333 µl of 2% formic acid in methanol (v/v). A heated vacuum centrifuge was used for reducing the samples to dryness. After reconstitution to a total volume of 100 µl using 10% acetonitrile in 0.1% formic acid (v/v), samples were filtered into a 96-well receiving plate through a poly tetrafluoro ethylene filter plate (0.45 µm; ChromTech, Apple Valley, MN). The receiving plate was then loaded into an Acquity liquid handling system (Waters Corp., Milford, MA) connected to liquid chromatography (LC).

A Luna C18(2) column (3 µm, 2.1 mm x 150 mm, Phenomenex, Torrance, CA) was utilized for LC separation. The flow rate was 0.2 ml/min); the injection volume was 50 µl; the mobile phase was acetonitrile (20% v/v) in 0.1% formic acid (80% v/v. The LC system was coupled with a Quattro Premier tandem quadrupole mass spectrometer (Waters Corp.) that had electrospray ionization. Source block temperature was set at 100 °C and desolvation temperature at 300 °C. The desolvation gas was Nitrogen gas (750 l/hr); the collision gas was Argon. The pressure of the collision cell was set at 3.8 x 1023 mBar. Positive ions were measured in multiple reaction monitoring mode for the transitions of MPD (mass-to-charge ratio, m/z 234 > m/z 84) and MPD-d9 (m/z 240 > m/z 89). Both quadrupoles operated at a resolution of 0.9 thomson (a unit of mass-to-charge ratio). For all MPD transitions, the experimenters used cone voltage of 30 V and collision energy of 20 eV.

Deuterium-labeled MPD-d9 was used as the internal standard. Characterization of MPD-d9 was performed using LC with electrospray mass spectrometry (MS) in the full scanning mode (m/z

100–600). The d6 isomer was used for all quantitations since the major isotopic peak corresponded to this species. Solid unlabeled MPD was carefully weighted to prepare a reference standard for analyzing the concentration of the labeled MPD internal standard in LC-UV (254 nm). This analysis showed that 1.4% of the internal standard was unlabeled. A standard curve was constructed using seven concentrations of unlabeled MPD standards (2–100 pg, duplicate injections) in the presence of 40 pg of labeled MPD internal standard. The plot was linear ($r2 > 0.999$) with a slope (response factor) of 9.15. An amount of 0.1 ng/ml was subtracted from each sample to account for the unlabeled MPD in the internal standard.

Brain Tissue: MPD concentrations in brain tissue were determined using a MPD direct enzyme-linked immunosorbent assay (ELISA) kit (Immunalysis, Pomona, CA). Frozen samples were weighed in tared tubes and homogenized with weight x 2.54 ml/g of 1X Phosphate Buffer Saline. The homogenate was centrifuged at 35,000xg at 4 °C for 20 min. 10 µl of the supernatant was added to each well. ELISA was performed according to the manufacturer's instructions and incorporating a methylphenidate standard curve. Optical density of MPD was measured with a photometer for absorbance at 405 nm.

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