Early postnatal treatment effect of methylphenidate on spontaneous and reward alteration and neuronal morphology of hippocampus in rats

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Abstract

Background: Attention Deficit Hyperactivity Disorder (ADHD), is one among the most common neuropsychiatric disorders in children. It is associated with other mental illness like anxiety and mood related disorders, which can continue into adulthood. Methylphenidate (MPH) is the primary choice of treatment, and has been so for many years. MPH is effective in relieving the symptoms of ADHD. The early exposure effects of MPH on cognition are of concern, since this drug is inappropriately used in paediatrics. The present study examines the long-term effect of early exposure of MPH on the spatial memory, and histomorphological changes in the hippocampus of normal rats. Methods: Rats received 2 mg/kg or 5 mg/kg dose of MPH or saline during postnatal days 7-35. Rats were subjected to a battery of cognitive tests on postnatal day 90 (PND90). The histomorphological study of various regions of the hippocampus and dentate gyrus were performed on postnatal day 90 or 135. Results: In the cognitive function test, the mean percentage of correct response was not altered in MPH treated rats as compared to their normal untreated counterparts, but the spontaneous alteration test revealed an improvement in learning ability during adulthood. At PND90, the neuronal loss was confined to CA4 hippocampal region and the dentate gyrus, but at PND135, there was significant neuronal loss in CA3, CA2, CA1 regions but not in the dentate gyrus. Conclusion: Early exposure to MPH in rats can adversely affect the survival of the adult neurons. Further, sensitive cognitive tests during adulthood is required to substantiate the neuronal loss observed in this study.

Key words: Attention deficit hyperactivity disorder, hippocampal neurons, dentate gyrus

Introduction

Attention Deficit Hyperactivity Disorder (ADHD), is a disease in children and adolescents characterized by inappropriate levels of inattention, hyperactivity, and/or impulsivity. These symptoms can lead to learning disabilities in school. The prevalence rate is increasing worldwide, affecting five percent of the child population. At times, ADHD conditions demand medical intervention, as parents and teachers in schools, find it difficult to handle these children. Those children who meet the diagnostic criteria of ADHD, are often prescribed psychostimulants...
by paediatricians and paediatric psychologists. Methylphenidate (MPH), is one among the most popular drugs, which is advised for the symptoms of ADHD and is proven to be effective in reducing hyperactivity and improving attentiveness. MPH can affect the development of the brain if it is given early in life.

MPH is known to be an inhibitor of dopamine and norepinephrine transporter. Juvenile administration of MPH to rodents is known to cause behavioural changes and a loss of dopaminergic neurons. Long-term treatment with MPH is known to enhance cell proliferation and neuronal differentiation. The studies have revealed an increased level of dopamine in the nucleus accumbens, and higher level of norepinephrine in the prefrontal cortex (PFC) and the hippocampus after MPH treatment in rats. Both norepinephrine (NE) and dopamine (DA) have a critical influence on PFC and hippocampus dependent cognitive functioning.

Materials and Methods

Animals: Inhouse bred male seven-day old albino *wistar* rats (19±2gm) were used in the present study. Rats were fed with water and food *ad libitum*. The rats were maintained under standard laboratory conditions. Institutional animal ethics committee approval was obtained before commencing the experiment.

Drug: MPH is available as Addwize (Sun Pharmaceutical Industries Ltd., India) 5 mg and 10 mg in the Indian market. In our study, the drug was administered intraperitoneally.

Animal groups

The experiment consisted of the following animal groups (number of rats in each group, n=8).

Group 1: Control rats received equal volume of i.p. saline

Group 2: Rats received 2 mg/kg MPH twice daily from postnatal day 7 (PND7) to 35 (PND35)

Group 3: Rats received 5 mg/kg MPH twice daily from PND7 to PND35

MPH powder thoroughly mixed with saline administration (intraperitoneal) began from PND7, because at this point of time the first adult like neurons are observed in the rat dentate gyrus, and neuronal growth and synaptogenesis actively begins. MPH administration continued until PND35 because at this age the maturation level of the brain corresponds to that observed in adolescent humans. All rats were subjected to behavioral studies at PND 90, and thereafter four rats from each group were sacrificed for histological studies. The remaining four rats from each group were sacrificed on PND 135 for histomorphological studies to evaluate long-term effects.

Behavioral studies

1. **Open field test:** Open field test is one of the most widely used methods to assess the motor and exploratory activities and emotional reactivity of rodents. The apparatus and procedures were similar to the one used before.

2. **Spatial learning test (T-maze test):** To assess the spatial learning ability, rats were subjected to spontaneous alternation and
rewarded alternation tests. The T-Maze test was conducted according to the methods used earlier by Rai et al.\textsuperscript{15}

i) Spontaneous alternation test: During these sessions, 15 pellets of food (10 mg each) were kept in each goal area. On the following four days, six trials were given daily. In each trial, the rat was placed in the start box and the door opened, thus allowing it to enter into the stem and arms of the T-maze. After the rats ate the pellet in the goal area, it was replaced back in the start box. In each trial, the arm chosen by the rat and number of alternations made, were noted. The inter-trial interval was one minute. The rat was deemed to have entered into a particular arm, when it entered that arm with all its four limbs. Percentage bias was calculated for each rat using the following formula:

More number of alternations and less percentage bias was considered as an index of improved learning ability.

ii) Reward alternation test: This test was done after completion of spontaneous alternation test. The test consisted of six trials/day, for four consecutive days. Each trial had two runs viz. forced run and choice run. In the forced run, the rat was forced to one of the arms by blocking the other arm and allowing it to consume the pellet there. In the choice run, the forced arm is kept empty and the pellet was placed in the opposite arm. Both the arms were free for the rat to run. Now, the rat had to enter into the arm opposite to the forced arm if it had to be considered as a “correct response.” The forced arm was predetermined, and it was same for all rats on any given day. It was changed on subsequent days. The experiment was repeated on four successive days. “Percentage of correct responses” was calculated for each rat by using the following formula:\textsuperscript{15}

Increase in mean percentage correct response was considered as improved learning and memory.

3. Histomorphological study:

Perfusion: Rats were deeply anesthetized and transcardially perfused with 0.9 percent saline, followed by 10 percent formalin at the same flow rate. The animal was decapitated and the brain was removed and kept in 10 percent formalin for 48 hours (post fixation). Paraffin blocks were made in an embedding bath. Coronal sections of 5 μm thickness were cut using a rotary microtome. Six sections from each animal were mounted serially on air-dried gelatinized slides.

Neuronal assay using cresyl violet: Ten sections were stained with cresyl violet stain. One hundred milligrams of cresyl violet was dissolved in 100 ml of distilled water. To this 0.5 ml of 10 percent acetic acid was added to give a pH of 3.5-3.8. The stain was filtered before use.\textsuperscript{16}

Quantitative analysis was performed under light microscopy (40 X). For quantification of normal neurons in dentate gyrus region, 400 X 400 micron area was selected and in the different regions of the hippocampus (cornua amonis areas -CA1, CA3, and CA4) for about 400 μm length area was selected. The slides were screened using a Nikon trinocular microscope (H600L) under 40 X magnification. Neurons were quantified using imaging software NIS Elements Br version 4.30.

Statistical analysis: The data was expressed as mean ± SEM. The significance of differences among the groups were assessed using one-way analysis of variance (ANOVA) test, followed by Bonferroni’s multiple comparison post hoc test. P values <0.05 was considered significant. Graph pad version 3 was used for statistical analysis.

Results

1. Open field test: The total number of crossings were reduced significantly (p<0.05) in rats who received 2 mg/kg dose of MPH, as compared to control but not in rats who received 5 mg/kg of test drug. However, the rats who received 5 mg/kg dose of MPH showed a significantly
higher number of line crossings in open field apparatus compared to MPH-2 group of rats (Figure 1). The number of central square crossings did not differ among various groups (Figure 2). The number of peripheral square crossings were reduced significantly (p<0.05) in rats who received 2 mg/kg dose of MPH compared to control group (Figure 1). The rearing and grooming activities were not statistically affected by MPH treatment.

**2. T-maze test:** There was no significant difference (P=0.8535, F=0.1596) in mean number of alterations among the different groups tested (Figure 3). The mean percentage of bias data showed rats treated with MPH 2 mg/kg dose had less percentage bias (p<0.05) as compared to control. However, rats treated with MPH 5 mg/kg did not show any such reduction in percentage of bias. There was a reduction in the mean number of correct response (p<0.05) in rats treated with MPH 2 mg/kg, but not in MPH 5 mg/kg treated animals (P = 0.0151, F = 5.02) when compared to control group (Figure 4). The mean percentage of correct response did not show any difference among the groups (p = 0.0914, F = 2.627).

**3. 3A. Histomorphological changes in Hippocampus and Dentate gyrus at PND-95:** The neuronal density in CA1, CA2 and CA3 regions of the hippocampus was not affected by MPH treatment when evaluated on postnatal day 95. CA4 region of the hippocampus showed a significant (p<0.01) loss of neurons at 2 mg/kg dose of MPH, compared to control, but not at MPH 5 mg/kg dose (Figure 5).

The dentate gyrus also showed a significantly (p<0.05) reduced neuronal density in rats treated with 2 mg/kg, but not 5 mg/kg of MPH. There was no significant difference in neuronal density.
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between 2 mg/kg and 5 mg/kg dose of MPH in all the regions investigated (Figure 5, Figure 6 and Figure 7).

Figure 5: Mean number of neurons in hippocampus (400 µm length area) and dentate gyrus (400 µm² area) region of the rats. Values are expressed as mean ± SE, Comparison between Control Vs MPH, * = p<0.05 ** = p<0.01, (n=8).

3B. Histomorphological changes in Hippocampus and Dentate gyrus at PND-135:
Except the CA1 region of the hippocampus, all the other regions of the hippocampus were affected by MPH treatment when evaluated at PND 135. In the CA2 region, MPH in a dose of 2 mg/kg caused significant (p<0.01) reduction in neuronal density, as compared to control and higher dose of MPH (p<0.05). In CA3 and CA4 regions, both groups treated with MPH showed a highly significant (p<0.01) loss of neurons as compared to control. However, no difference in effect in the CA3 region was observed between MPH treated groups. The neuronal loss in the CA4 region was more severe in MPH-5 mg/kg treated group (p<0.001). The neurons of the dentate gyrus were not affected by MPH treatment (Figure 8).

Figure 6: Photomicrograph of CA4 region of the hippocampus stained with cresyl violet under 40X magnification. Scale bar indicates 400 µm length area. Note the distorted neurons in MPH-2 group.

Figure 7: Photomicrograph of dentate gyrus region of the rat brain, stained with cresyl violet under 40X magnification. Square area represents 400 µm² area.

Figure 8: Mean number of neurons in hippocampus (400 µm length area) and dentate gyrus (400µm² area) region of the rats. Values are expressed as mean ± SE, Comparison between Control Vs. MPH, ** = p<0.01 *** = p<0.001, $=p<0.05, (n=8).
Discussion

MPH is found to be effective in tackling ADHD for many years.\(^1\) The effects of MPH in children and animal models on a short-term basis has been established. However, there is scanty information about its long-term effects on the developing brain. MPH treatment mainly blocks dopamine transporters, and to the lesser extent norepinephrine and serotonin transporters.\(^2\) MPH induced altered expression of these brain monoamines during brain development can have many neurological consequences in the later part of life. It is established that juvenile MPH treatment affects synaptogenesis, myelination and gliogenesis.\(^17\) The frontal cortex and the hippocampal region, which are involved in the learning and cognition, exhibited more pronounced effects of MPH. To study these effects, the present study used a rat model which mimics the pediatric therapeutic doses of MPH.

The results of the open field test, demonstrate that MPH treatment at 2 mg/kg dose has enhanced locomotor activity. However, reduced peripheral square crossings in MPH-2 group did not show corresponding elevation in central square crossings. Hence, it would not be appropriate to conclude that MPH treatment would cause anxiety like behavior or an anxiolytic action in adult rats. Vendruscolo et al.,\(^18\) reported that, MPH given in the adolescent rats resulted in anxiety like behavior, later in the adult life of the rat. Several recent studies also showed that an early exposure to MPH would lead to anxiety disorder in adult rats.\(^19\)\(^,\)\(^20\)\(^,\)\(^21\) A more sensitive study model, like elevated plus maze, would provide a better picture regarding anxiety like behavior, because open field test basically tests emotion. The rats are prone to avoid the central part and concentrate more in the periphery of the given new area, which is quite larger. This suggests that, the quantity of central square crossings is the index of anxiety due to the experiment in this open field test, while the locomotion is being represented by the number of peripheral line crossings.\(^22\)

In the spontaneous alteration test, though mean number of alterations were not increased in MPH treated rats, the percentage of bias was reduced in adult rats who received 2 mg/kg dose during the juvenile period, compared to their control counterparts. This is an index of improved learning ability. The rats treated with MP 2 mg/kg dose had less percentage bias compared to control. The mean number of correct response showed a reduction in MP 2 mg/kg treated rats but not in MP 5 mg/kg treated rats when both treated groups were compared to control group of rats. Even the mean percentage of correct response did not show any difference among the groups. Hence, the spatial memory test does not clearly confirm any (either beneficiary or adversary) effect on cognition. In contrast to our finding, the data published by Rostron et al.,\(^23\) suggested a detrimental effect during task performance after MPH exposure. MPH, being DA agonist, was tested for its cognitive enhancement ability after traumatic brain injury. An improvement in memory acquisition and retention was observed in male rats but not in females. This study\(^23\) suggested that, female sex hormones modulate the dopamine transport system. But in the present study, though gender related effect of MPH was not focused upon, it would be worth considering such studies in future.

Results have been inconsistent regarding neuronal proliferation and differentiation in young rats after juvenile MPH treatment. MPH (10 mg/kg dose) for 28 consecutive days enhanced neuronal proliferation and neuroblast differentiation.\(^3\) But, Lagace and his co-workers\(^25\) observed that MPH at 2 mg/kg dose for 16 days of treatment, reduced the survival of matured hippocampal neurons in adult rats. With respect to the contrasting results observed in these in vivo studies, an in vitro study by Jasmin Bartl\(^26\) observed a decrease in neuronal proliferation and an increase in neuronal differentiation. For neuronal maturation, it is required that the neuronal stem cells stop proliferating, so that they can start the outgrowth of the neuritis to develop into a full neuron. However, the findings of this recent in vitro study is not consistent with an earlier in vivo study by Diane et al.\(^27\) In this study, early (PND20-35) MPH (2 mg/kg dose) exposure did not alter neuronal proliferation when tested at various postnatal days (PND90 and PND112), but affected the survival of adult neurons adversely. Interestingly, in the present
study we observed early postnatal MPH treatment has caused a loss of neurons in dentate gyrus (an area actively involved in neurogenesis even during postnatal life) and also CA4 region of the hippocampus at PND95, but not in the other regions of the hippocampus. Interestingly at PND135, a loss of neurons was observed more in regions of the hippocampus that includes CA2, CA3, and CA4. The mechanisms involved in this delayed onset of loss of neurons in the hippocampus have to be further addressed. Our study supports the work by Diane et al., and Lagace, in which they observed the failure of adult neurons to survive. Though this study did not focus on neuronal proliferation, it is clear that MPH treatment reduced adult (PND135) neuronal population in the hippocampus. Further, dentate gyrus where active neurogenesis occurs even during adulthood, did not show statistically significant loss of neurons (at both PND90 and 135); this could be due to continuous formation of new sets of neurons. In another recent study by Vrinda et al., MPH (2 or 5 mg/kg dose) treatment from PND23 TO PND45, resulted in considerable neuronal loss at the medial side of the prefrontal cortical area of the rats during adulthood. MPH treatment mainly blocks dopamine transporters and to the lesser extent norepinephrine. The correct functioning of the decision making depends on the maintenance of balance between the dopaminergic and noradrenergic system, and the prefrontal cortex is crucial for it. The ventral tegmental area provides the dopaminergic input to the prefrontal cortex and the MPH has been reported to increase the dopamine availability in this topography. The MPH simultaneously improves the cognitive performance in the ADHD patients. It would be more appropriate to evaluate the DA and NE levels or immunoreactivity for these neurons at various postnatal periods after juvenile MPH treatment, as this could provide the basis for loss of matured neurons in the areas like the hippocampus and prefrontal cortex. Sadasivan et al. attempted to observe the chronic effects of MPH at a dose of 10 mg/kg body weight. This reduced dopaminergic neurons in the substantia nigra in mice, which was associated with an increase in activated microglia. Though MPH is known to exert good results in children with ADHD, its adverse effect on survival of adult neurons is of concern, in spite of its good results in children with ADHD.

Conclusion

Since MPH therapy often begins at the time when the brain is still developing, its consequences are of concern for public health due to possible adverse effects on behavior. We believe that the present study has provided additional information in comparison to the previous studies about this subject. The present study observed that, the early exposure to MPH during the childhood and adulthood can result in adverse effects, which are obvious later in the adult life. Interestingly, our study did not show any effect of MPH treatment on cognition in rats unlike many other studies, but certainly caused neuronal loss during adulthood. The loss of neurons during early adulthood is of serious concern. Though the neurogenesis continues during early adulthood and other compensatory mechanism does exists, still it is a matter to be investigated further. Of late, there is increased usage of MPH in individuals who are not meeting the criteria of ADHD. MPH is also misused as an alternative to the psychostimulants and cognitive enhancers. Exposure to MPH during the early stages of brain development raises serious concerns for public health.

References

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