Research report

Stimulation of postsynapse adrenergic $\alpha_{2A}$ receptor improves attention/cognition performance in an animal model of attention deficit hyperactivity disorder

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HIGHLIGHTS

- Clonidine improved attention deficit in the 5-trial inhibitory avoidance model.
- Guanfacine improved attention deficit in the 5-trial inhibitory avoidance model.
- Adrenergic $\alpha_{2A}$ receptor stimulation mediated the effects of both compounds.
- A selective noradrenergic neurotoxin did not affect the effects of both compounds.

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ABSTRACT

A 5-trial inhibitory avoidance test using spontaneously hypertensive rat (SHR) pups has been used as an animal model of attention deficit hyperactivity disorder (ADHD). However, the roles of noradrenergic systems, which are involved in the pathophysiology of ADHD, have not been investigated in this model. In the present study, the effects of adrenergic $\alpha_{2}$ receptor stimulation, which has been an effective treatment for ADHD, on attention/cognition performance were investigated in this model. Moreover, neuronal mechanisms mediated through adrenergic $\alpha_2$ receptors were investigated. We evaluated the effects of both clonidine, a non-selective adrenergic $\alpha_2$ receptor agonist, and guanfacine, a selective adrenergic $\alpha_{2A}$ receptor agonist, using a 5-trial inhibitory avoidance test with SHR pups. Juvenile SHR exhibited a shorter transfer latency, compared with juvenile Wistar Kyoto (WKY) rats. Both clonidine and guanfacine significantly prolonged the transfer latency of juvenile SHR. The effects of clonidine and guanfacine were significantly blocked by pretreatment with an adrenergic $\alpha_{2A}$ receptor antagonist. In contrast, the effect of clonidine was not attenuated by pretreatment with an adrenergic $\alpha_{2B}$ receptor antagonist, or an adrenergic $\alpha_{2C}$ receptor antagonist, while it was attenuated by a non-selective adrenergic $\alpha_2$ receptor antagonist. Furthermore, the effects of neither clonidine nor guanfacine were blocked by pretreatment with a selective noradrenergic neurotoxin. These results suggest that the stimulation of the adrenergic $\alpha_{2A}$ receptor improves the attention/cognition performance of juvenile SHR in the 5-trial inhibitory avoidance test and that postsynaptic, rather than presynaptic, adrenergic $\alpha_{2A}$ receptor is involved in this effect.

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1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by hyperactivity, inattention and impulsivity that affects 8–12% of children [1]. Although the etiology and pathophysiology of ADHD are not fully known, dysfunctional dopaminergic and noradrenergic systems are thought to play key roles [2]. Although stimulants, such as methylphenidate and amphetamine, have been regarded as effective pharmacological treatments, there are several drawbacks to the current medications that are available, including the existence of treatment-refractory patients and side effects, such as the potential for abuse [3]. Thus, there is a need for alternatives to the current medications, and a suitable and pharmacologically validated animal model is necessary to achieve this goal.

Fox et al. [4] reported the 5-trial inhibitory avoidance test using juvenile spontaneously hypertensive rats (SHR), which have been shown to exhibit abnormalities of catecholaminergic
neural activity [5] and several major ADHD-like behaviors such as impulsivity, hyperactivity, and poor sustained attention [6,7], as an animal model for ADHD. In this test, juvenile SHR exhibited a significantly impaired performance, compared with normotensive juvenile Wistar Kyoto (WKY) rats, upon repeated exposure to mild aversive stimuli, such as a short duration (1 s) and a weak current (0.1 mA); methylphenidate, a prescribed and effective treatment for ADHD, improved the impaired performance of juvenile SHR when examined using this test [4,8]. Thus, the 5-trial inhibitory avoidance test using juvenile SHR may represent impaired attention/cognition and impulsivity, which are similar to the symptoms observed in patients with ADHD, and this model may be useful for evaluating the effects of compounds on attention/cognition and impulsivity. However, pharmacological validation of the 5-trial inhibitory avoidance test has not been fully conducted, and the possible involvement of noradrenergic pathways, which have been proposed to have an important role in attention and impulsivity, in this model remains unknown.

Recently, adrenergic α2 receptor stimulation has been reported to be an effective means of treating ADHD. Indeed, the efficacies of both clonidine, a non-selective adrenergic α2 receptor agonist, and guanfacine, a selective adrenergic α2 receptor agonist, for the treatment of ADHD, including impaired attention and impulsivity, have been demonstrated in clinical settings [9–12]. However, the roles of adrenergic α2 receptors in the 5-trial inhibitory avoidance test have not been investigated. In addition, although the involvement of the α2A receptor in attention/cognition and impulsivity has been well acknowledged [5,13], the roles of other adrenergic α2 receptor subtypes, particularly α2B and α2C receptors, in the actions of clonidine have not been addressed. Therefore, in the present study, we evaluated the effects of clonidine and guanfacine in the 5-trial inhibitory avoidance test using juvenile SHR. Moreover, because α2 receptors are expressed both presynaptically and postsynaptically, some of the neuronal mechanisms mediated through the stimulation of adrenergic α2 receptors were investigated.

2. Methods

2.1. Animals

Male WKY rats and SHR (4 weeks old at time of the experiment) were purchased from Charles River (Yokohama, Japan). All the rats were housed in plastic cages under a 12-h light:12-h dark cycle (lights on at 07:00 h). The rats were also maintained under controlled temperature (23 ± 3°C) and humidity (50 ± 20%). Food and water were available ad libitum. All the experiments were conducted in accordance with the criteria of the Taisho Pharmaceutical Co., Ltd. Animal Care Committee and met the Japanese Experimental Animal Research association standards, as defined in the Guidelines for Animal Experiments.

2.2. 5-Trial inhibitory avoidance test

The 5-trial inhibitory avoidance test was performed according to a previously described method [4,8], with certain modifications. Rats were examined in a step-through type cage (Muromachi Kikai Co., Ltd., Tokyo, Japan), which consisted of a bright compartment (width: 250 mm, depth: 210 mm, height: 170 mm; light level: about 110 lx), a dark compartment (width: 250 mm, depth: 210 mm, height: 170 mm) and a grid floor. The two compartments were separated by a plastic door (width: 50 mm, height: 50 mm). A rat was placed in the bright compartment. Then, the transfer latency until entering the dark compartment was measured using a stopwatch. Once the rat had transferred to the dark compartment, the door was closed and an electric shock (0.1 mA, 1 s) was applied through the grid using a shock generator (Muromachi Kikai Co., Ltd., Tokyo, Japan). After the application of the foot-shock, the rat was returned to the home cage. The process was then repeated for five trials, with an inter-trial interval of 1 min. The apparatus was cleaned with 70% ethanol between trials. The cut-off times were 60 s for trial 1 and 180 s for the other trials. The rats that entered into the dark compartment within the cut-off time of 60 s in trial 1 were selected for trials 2–5. When the transfer latency was beyond the cut-off time in trials 2–5, 180 s was registered as the result. The measurements were conducted in a blinded fashion.

2.3. Measurement of sensitivity to foot-shock

This experiment was conducted to investigate whether or not methylphenidate, clonidine, and guanfacine exerted non-specific effects, such as a perception alteration of the foot-shock. This test was performed according to a previously described method [4], with certain modifications. A rat was placed into the dark compartment under a condition that allowed the door between the two compartments to remain closed. The inescapable foot-shock current was gradually ramped up from 0.05 mA to 0.4 mA, and then gradually back to 0.05 mA over a period of 30 s. iMax and iMin were defined as the first current when the rats vocalized and when the rats ceased vocalization, respectively.

2.4. Measurement of noradrenaline concentration in the prefrontal cortex in SHR

To confirm that the noradrenaline level is depleted by N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4) in the prefrontal cortex, the noradrenaline level was measured using an enzyme-linked immunosorbent assay (ELISA) (Noradrenaline Research ELISA™; Labor Diagnostika Nord GmbH and Co. KG, Nordhorn, Germany). Rats were sacrificed by decapitation, and the prefrontal cortex (cingulate cortex and infralimbic area in Fig. 8 and Fig. 9 in a rat brain atlas [14]) was dissected bilaterally from a 4-mm-thick coronal section of the anterior pole of the brain. The tissues were homogenized in 0.01 M hydrochloric acid containing 4 mM sodium metabisulphite and 1 mM ethylenediamine tetraacetic acid. After centrifugation (17,600 × g, 10 min, 4°C), 5 µL of the supernatant was applied to an ELISA to measure the noradrenaline concentration.

2.5. Drugs and treatments

Clonidine hydrochloride, guanfacine hydrochloride, yohimbine hydrochloride, 2-[[4,5-dihydro-1H-imidazol-2-yl][methyl]-2,3-dihydro-1-methyl-1H-isoindole maleate (BRL44408), imiloxan hydrochloride, and DSP-4 hydrochloride were produced from Sigma–Aldrich (St. Louis, MO, USA). Methylphenidate hydrochloride was synthesized at Taisho Medicinal Research Laboratories. Acidin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine dihydrochloride (JP-1302) was purchased from Abcam (Cambridge, UK). All drugs except for yohimbine were dissolved in saline. Yohimbine was dissolved in distilled water. Clonidine, methylphenidate and guanfacine were injected subcutaneously 30 min before the 5-trial inhibitory avoidance test and the measurement of the foot-shock response. Yohimbine and BRL44408 were injected intraperitoneally 15 and 20 min before the injection of clonidine or guanfacine, respectively. Imiloxan and JP-1302 were injected subcutaneously 20 and 60 min before the injection of clonidine, respectively. DSP-4 was injected intraperitoneally 3 days before the 5-trial inhibitory avoidance test and the measurement of the prefrontal cortical noradrenaline contents. The drug administrations, except for yohimbine and BRL44408, were performed in
2.6. Statistical analysis

All the statistical analyses were performed using SAS software (SAS Institute Japan, Tokyo). Data from the behavioral experiments were analyzed using Wilcoxon’s test or Kruskal–Wallis followed by the Steel’s or Steel–Dwass post hoc test. Student’s t-test was used to examine the noradrenaline concentration. A value of $p < 0.05$ was regarded as significant.

3. Results

3.1. Performances of juvenile SHR and WKY rats in the 5-trial inhibitory avoidance test

Juvenile SHR exhibited a shallower learning curve and a shortened transfer latency, compared with juvenile WKY rats, in trials 3–5 (Fig. 1A). The juvenile SHR also exhibited a shorter total transfer latency for trials 2–5, compared with juvenile WKY rats (Fig. 1B).

3.2. Effect of methylphenidate on performances of juvenile SHR and WKY rats in the 5-trial inhibitory avoidance test

Methylphenidate (1 and 3 mg/kg, s.c.) prolonged the transfer latency in juvenile SHR (Fig. 2A), while it did not affect the transfer latency in juvenile WKY rats (Fig. 2B), in the 5-trial inhibitory avoidance test. Methylphenidate (3 mg/kg, s.c.) did not alter the sensitivity to foot-shock in either the juvenile SHR or the juvenile WKY rats (Fig. 4A and B). In the absence of foot-shock, methylphenidate (3 mg/kg, s.c.) did not affect the transfer latency in the juvenile SHR (vehicle: $25.2 \pm 11.8$ s/T2–T5, methylphenidate: $30.5 \pm 8.0$ s/T2–T5, $n = 6$ per each group, $p > 0.05$).

3.3. Effects of clonidine and guanfacine on performances of juvenile SHR and WKY rats in the 5-trial inhibitory avoidance test

A non-selective adrenergic $\alpha_2$ receptor agonist, clonidine (0.01 and 0.03 mg/kg, s.c.), prolonged the transfer latency in juvenile SHR (Fig. 3A), while it did not affect the transfer latency of juvenile WKY rats (Fig. 3B) in the 5-trial inhibitory avoidance test. Juvenile SHR reduced both iMax and iMin (Fig. 4A and B). In contrast, clonidine (0.03 mg/kg, s.c.) did not alter the sensitivity to foot-shock in either the juvenile SHR or the juvenile WKY rats (Fig. 4A and B), with the exception that the iMax in the juvenile SHR increased significantly.

Fig. 1. Performance of juvenile SHR and WKY rats in the 5-trial inhibitory avoidance test. (A) Learning curve in the 5-trial inhibitory avoidance test. (B) Total transfer latency of trials 2–5. Data are expressed as the mean ± S.E.M., $n = 10–12$ animals per each group. *$p < 0.05$, **$p < 0.01$, compared with the response to WKY. WKY: WKY rats.

Fig. 2. Effect of methylphenidate on performance of juvenile SHR (A) and WKY rats (B) in the 5-trial inhibitory avoidance test. Data are expressed as the mean ± S.E.M., $n = 10–12$ animals per each group. *$p < 0.05$, **$p < 0.01$, compared with the response to vehicle in SHR. WKY: WKY rats.
(Fig. 4A). Therefore, juvenile SHR may show higher sensitivity to foot-shock compared with juvenile WKY rats, and clonidine may exert an antinociceptive-like action in juvenile SHR. In the absence of foot-shock, clonidine (0.03 mg/kg, s.c.) did not affect the transfer latency in juvenile SHR (vehicle: $25.2 \pm 1.18$ s/T2–T5, clonidine: $43.3 \pm 12.6$ s/T2–T5, $n=6$ per each group, $p>0.05$). A selective adrenergic $\alpha_{2A}$ receptor agonist, guanfacine (0.3 and 1 mg/kg, s.c.), significantly prolonged the transfer latency of juvenile SHR (Fig. 5A), while guanfacine (1 mg/kg, s.c.) did not alter the sensitivity to foot-shock (Fig. 5B). In the absence of foot-shock, guanfacine (1 mg/kg, s.c.) also did not affect the transfer latency in juvenile SHR (vehicle: $25.2 \pm 1.18$ s/T2–T5, guanfacine: $26.8 \pm 6.7$ s/T2–T5, $n=6$ per each group, $p>0.05$).

3.4. Involvement of adrenergic $\alpha_{2A}$ receptor in the effect of clonidine

Pretreatment with yohimbine (2 mg/kg, i.p.) (Fig. 6A) and BRL44408 (3 and 10 mg/kg, i.p.) (Fig. 6B) significantly blocked the prolonged transfer latency of juvenile SHR induced by clonidine (0.03 mg/kg, s.c.) in the 5-trial inhibitory avoidance test. The effect of guanfacine (1 mg/kg, s.c.) on the transfer latency in the 5-trial inhibitory avoidance test was also blocked by BRL44408 (10 mg/kg, i.p.) (Fig. 6C). Pretreatment with imiloxan (10 mg/kg, s.c.) (Fig. 7A) and JP-1302 (3 mg/kg, s.c.) (Fig. 7B) did not block the prolonged latency of juvenile SHR induced by clonidine (0.03 mg/kg, s.c.) in the 5-trial inhibitory avoidance test. Yohimbine (2 mg/kg, i.p.), BRL44408 (3 and 10 mg/kg, i.p.), imiloxan (10 mg/kg, s.c.), and

Fig. 5. Effect of guanfacine on performance in the 5-trial inhibitory avoidance test (A) and on foot-shock response (B) in juvenile SHR. Data are expressed as the mean±S.E.M., n = 6–7 animals per each group. *p < 0.05, compared with the response to vehicle.

Fig. 6. Effects of yohimbine (A) and BRL44408 (B and C) on the prolonged transfer latency induced by clonidine (A and B) or guanfacine (C) in juvenile SHR. Data are expressed as the mean±S.E.M., n = 8–12 animals per each group. *p < 0.05, **p < 0.01, compared with the response to vehicle. + vehicle. #p < 0.05, compared with the response to vehicle + clonidine. $$$p < 0.01, compared with the response to vehicle + guanfacine.
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Fig. 7. Effects of imiloxan (A) and JP-1302 (B) on the prolonged transfer latency induced by clonidine in the 5-trial inhibitory avoidance test in juvenile SHR. Data are expressed as the mean ± S.E.M., n = 9–12 animals per group. **p < 0.01, compared with the response to vehicle + vehicle. ***p < 0.01, compared with the response to imiloxan + vehicle.##p < 0.01, compared with the response to JP-1302 + vehicle.

JP-1302 (3 mg/kg, s.c.) themselves did not affect the transfer latency of juvenile SHR (Figs. 6 and 7).

3.5. Involvement of postsynaptic adrenergic α2A receptor in the effects of clonidine and guanfacine

Treatment with DSP-4 (50 mg/kg, i.p.) decreased the noradrenaline level in the prefrontal cortex by approximately 60%, compared with the vehicle control, in juvenile SHR (vehicle: 182.9 ± 19.3 ng/g tissue, DSP-4: 71.5 ± 10.5 ng/g tissue, n = 4 per each group, p < 0.01). DSP-4 treatment (50 mg/kg, i.p.) did not affect the transfer latency, per se, or the prolonged transfer latency induced by both clonidine (0.03 mg/kg, s.c.) and guanfacine (1 mg/kg, s.c.) in the 5-trial inhibitory avoidance test in juvenile SHR (Fig. 8).

4. Discussion

We demonstrated, for the first time, that both clonidine and guanfacine improved the attention/cognition performance and impulsivity of juvenile SHR in the 5-trial inhibitory avoidance test and that the effects of both drugs may be mediated through the stimulation of postsynaptic adrenergic α2A receptor.

In this study, it was shown that juvenile SHR exhibited a shorter transfer latency, compared with juvenile WKY rats, and that a prescription drug for ADHD, methylphenidate, prolonged the transfer latency without affecting performance in the absence of foot-shock and the sensitivity to foot-shock in juvenile SHR. These results are consistent with previously reported results [4], and the present conditions should be useful for detecting the efficacy of compounds on impaired attention/cognition and impulsivity in this test. Of note, juvenile SHR showed higher sensitivity to foot-shock, which causes reluctance to enter the dark compartment. Therefore, shorter latency observed in juvenile SHR may reflect impaired attention and impulsivity but not increased pain threshold.

One of the objectives of the present study was to investigate the involvement of adrenergic α2 receptors in this test, and we found that another prescription drug for ADHD, clonidine, a non-selective adrenergic α2 receptor agonist, prolonged the transfer latency in juvenile SHR without affecting the transfer latency in juvenile WKY rats. In contrast, clonidine did not affect performance in juvenile SHR under the present conditions in the absence of foot-shock. Moreover, an adrenergic α2A receptor agonist, guanfacine, which has also been proven to be effective for ADHD, prolonged the transfer latency of juvenile SHR in the 5-trial inhibitory avoidance test. Of note, both compounds did not increase the response to foot-shock in SHR, while clonidine rather reduced sensitivity to foot-shock. Given that reduced sensitivity may shorten transfer latency, the effects of clonidine in the 5-trial inhibitory avoidance test are unlikely to be attributed to changes in sensitivity to foot-shock. Therefore, these results show that the stimulation of adrenergic α2 receptors improves impaired attention/cognition and impulsivity in the 5-trial inhibitory avoidance test using juvenile SHR, which is also underpinned by the report that methylphenidate enhance working memory performance in monkey by stimulation of adrenergic α2 receptor [21]. Moreover, the present results strengthen the pharmacological validity of the 5-trial inhibitory avoidance test as an animal model for ADHD.

Although the involvement of the adrenergic α2A receptor in attention/cognition impairment and impulsivity has been well acknowledged, the roles of other adrenergic α2 receptor subtypes, such as α2B and α2C receptors, particularly in the actions of clonidine, have not been fully investigated. Indeed, the involvement of adrenergic α2B and/or α2C receptors in the action of clonidine on prefrontal cortical function has been suggested [22], raising the possibility that both receptor subtypes may have some roles in the action of clonidine in the 5-trial inhibitory avoidance test as well. Moreover, while the adrenergic α2A receptor is distributed widely throughout the brain, with high levels in the prefrontal cortex, locus coeruleus, amygdala and hippocampus, both adrenergic α2B and α2C receptors are also expressed in brain regions that are involved in attention/cognition, such as the thalamus, striatum, hippocampus and cerebral cortex [23,24]. In the present study, both a non-selective adrenergic α2 receptor antagonist, yohimbine, and an adrenergic α2A receptor antagonist, BRL44408, blocked the effect of clonidine. BRL44408 also blocked the effect of guanfacine, suggesting that the dose of BRL44408 used in the present study was suitable for blocking the adrenergic α2A receptor in vivo. In contrast, neither an adrenergic α2B receptor antagonist, imiloxan, nor an adrenergic α2C receptor antagonist, JP1302, influenced the effect of clonidine. Imiloxan at a dose of 3 mg/kg blocked the antinociception of centhaquin, a centrally-acting anti-hypertensive drug, in mice [25], and JP-1302 at a dose of 3 mg/kg reduced immobility in the forced swimming test and reversed phencyclidine-induced prepulse inhibition deficits in rats [17]. Thus, the doses of imiloxan and JP-1302 used in the present study were sufficient to cause the pharmacological blockade of adrenergic α2B and α2C receptors, respectively. Therefore, the stimulation of the adrenergic α2A receptor is involved in the improvement of attention/cognition impairment and impulsivity in the 5-trial inhibitory avoidance test.
and both adrenergic \( \alpha_{2B} \) and \( \alpha_{2C} \) receptors are not involved in the actions of clonidine in this model.

The adrenergic \( \alpha_{2A} \) receptor is localized both pre- and postsynaptically in the prefrontal cortex [24], a brain region in which brain function is severely disturbed in patients with ADHD [26]. Moreover, the prefrontal cortical adrenergic \( \alpha_{2A} \) receptor has been suggested to have important roles in attention/cognition [27]. In the present study, treatment with a selective noradrenergic neurotoxin, DSP-4, markedly decreased the noradrenaline content by 60% in the prefrontal cortex of juvenile SHR. Under this condition, the effects of clonidine and guanfacine in the 5-trial inhibitory avoidance test were not affected. DSP-4 reportedly destroys noradrenergic terminals that originate in the locus coeruleus in brain regions such as the cortex and hippocampus [28]. Moreover, a reduction in the number of presynaptic adrenergic \( \alpha_{2} \) receptors [28] and the attenuation of presynaptic adrenergic \( \alpha_{2} \) receptor-regulated noradrenaline release [29] following DSP-4 treatment have been reported in the cortex and hippocampus. Therefore, the present results suggest that the effects of clonidine and guanfacine in the 5-trial inhibitory avoidance test may be mediated through postsynaptic, rather than presynaptic, adrenergic \( \alpha_{2A} \) receptors in the prefrontal cortex, although the involvement of a postsynaptic mechanism cannot be fully ruled out, considering the possible compensatory increase in noradrenergic transmission as a result of partial lesion with DSP-4 [30]. In addition, the roles of \( \alpha_{2A} \) receptor outside the prefrontal cortex need to be elucidated, because the degree of the reduction in the noradrenaline content as a result of DSP-4 treatment differs among brain regions.

The neural mechanisms underlying the activation of postsynaptic adrenergic \( \alpha_{2A} \) receptor have not been fully elucidated. Previous reports suggested that the stimulation of postsynaptic adrenergic \( \alpha_{2A} \) receptor in the prefrontal cortex has a beneficial effect on prefrontal cortex-dependent cognitive function [13,26,31]. The postsynaptic adrenergic \( \alpha_{2A} \) receptor was localized at the dendritic spines of pyramidal neurons in the prefrontal cortex of non-human primates [24]. Postsynaptic adrenergic \( \alpha_{2A} \) receptor reportedly enhances the activity of prefrontal pyramidal excitability in monkey, presumably through a reduction of cAMP, which leads to the closing of the hyperpolarization-activated cyclic nucleotide-gated channels [31]. Therefore, it is suggested that the regulation of the pyramidal neuronal activity by the stimulation of postsynaptic adrenergic \( \alpha_{2A} \) receptor in the prefrontal cortex may be involved in the action of clonidine and guanfacine on attention/cognition performance in the 5-trial inhibitory avoidance test.

In contrast, the stimulation of postsynaptic adrenergic \( \alpha_{2A} \) receptor has been reported to suppress excitatory synaptic transmission in the medial prefrontal cortex of rat [22]. Since suppression of transmission and enhancement of excitability are under respective spontaneous and task-related conditions, these discrepancies might be explained by differences in the experimental conditions. Therefore, further research is needed to prove our hypothesis.

In summary, the present study pharmacologically demonstrated that clonidine and guanfacine, both of which have been shown to be effective for the treatment of ADHD in clinical settings, improved the attention/cognition performance and impulsivity of juvenile SHR in the 5-trial inhibitory avoidance test, and that the stimulation of the postsynaptic adrenergic \( \alpha_{2A} \) receptor may be responsible for the effect of these drugs.

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