Aberrant CaMKII activity in the medial prefrontal cortex is associated with cognitive dysfunction in ADHD model rats.

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Abbreviations used: ADHD, Attention-deficit/hyperactivity disorder; AS, Angelman Syndrome; ATRX, α -thalassemia X-linked mental retardation; BDNF, brain derived neurotrophic factor; CaMKII, Ca^{2+} /calmodulin- dependent protein kinase II; cAMP, cyclic AMP; CREB, cAMP-responsible element-binding protein; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; DAT, Dopamine transporter; EPSC, excitatory postsynaptic current; ERK, extracellular signal-regulated kinase; GluR1, α -amino-3-hydroxy- 5-methyl-4-isoxazolpropionic acid-type glutamate receptor subunit 1; LTP, long-term potentiation; mPFC, medial prefrontal cortex; MPH, methylphenidate; NET, norepinephrine transporter; NMDA, N-methyl-D-aspartate; PFC, prefrontal cortex; PKA, protein kinase A; PKC, protein kinase C; PP, protein phosphatase; PPP2R2C, protein phosphatase 2 regulatory subunit B gamma; rCBF, regional cerebral blood flow; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto.

Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a heterogeneous neurobehavioral disorder accompanied by cognitive and learning deficits, which is prevalent among boys. Juvenile male stroke-prone spontaneously hypertensive rats (SHRSP) exhibit ADHD-like behaviors including cognitive deficits and represent one animal model of ADHD. Here, we define a mechanism underlying cognitive dysfunction observed in SHRSP. Acute methylphenidate (MPH: 1 mg/kg, p.o.) administration to SHRSP significantly improved not only inattention in a Y-maze task but also cognitive dysfunction in a novel object recognition test. Interestingly, Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) activity, which is essential for memory and learning acquisition, was excessively elevated in the medial prefrontal cortex (mPFC) but not in the hippocampal CA1 region of SHRSP compared with Wistar-Kyoto (WKY) rats. We also confirmed that elevated CaMKII autophosphorylation in the mPFC causes increased phosphorylation of the CaMKII substrate α -amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid-type glutamate receptor subunit 1 (GluR1) (Ser-831). Ca^{2+} -dependent phosphorylation levels of factors such as extracellular signal-regulated kinase (ERK) and protein kinase C (PKC) were unchanged in the SHRSP mPFC. Also, protein levels of the dopamine D2 receptor (D2R) but not the dopamine D1 receptor (D1R) were increased in the SHRSP mPFC. Acute MPH (1 mg/kg, p.o.) administration attenuated aberrant CaMKII activity and increased GluR1 phosphorylation observed in SHRSP. Taken together, we propose that cognitive impairment in SHRSP is associated with aberrant CaMKII activity in the mPFC.

Keywords

Attention-deficit/hyperactivity disorder, Ca²⁺/calmodulin-dependent protein kinase II, cognitive dysfunction, dopamine D2 receptor, stroke-prone spontaneously hypertensive rats

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is the most common, childhood-onset psychiatric disorder, affecting approximately 3-5% of school-aged children and frequently persisting into adulthood (Barkley, 1998; Szatmari et al., 1989). ADHD is a heterogeneous neurobehavioral disorder prevalent among boys and defined by three behavioral subtypes: predominantly inattentive, predominantly hyperactive and impulsive, and combined subtypes (American Psychiatric Association, 2000; Lahey et al., 1994). Additionally, neuropsychological studies suggest that ADHD patients exhibit cognitive dysfunction including impaired working memory (Tannock et al., 1995). ADHD is associated with loss of function of the prefrontal cortex (PFC), which plays a role in regulating complex cognitive, emotional, and behavioral activities (Castellanos and Tannock, 2002). In fact, ADHD-approved medications improve cognitive processes dependent on the PFC, including working memory, planning and control of impulsivity (Mehta et al., 2001; Turner et al., 2005). For example, methylphenidate (MPH), a first choice psychostimulant for ADHD, ameliorates not only inattention and hyperactivity-impulsivity but also cognitive dysfunction in ADHD patients by upregulating dopaminergic signaling in the PFC via inhibition of monoamine transporters (Conners and Eisenberg, 1963; Sykes et al., 1971; Tannock et al., 1995; Kempton et al., 1999). However, treatment of ADHD with psychostimulants increases abuse potential. To develop novel therapeutics lacking this risk, it is necessary to define the pathogenesis of ADHD.

The spontaneously hypertensive rats (SHR) (Okamoto and Aoki, 1963) have been widely used as an animal model of ADHD, as rats exhibit ADHD-like behaviors such as hyperactivity (Knardahl and Sagvolden, 1979; Wultz et al., 1990) and

impulsivity/inattention (Sagvolden et al., 1993; Sagvolden, 2000). However, SHRs do not fulfill all behavioral and pharmacological profiles of an animal model of ADHD. For example, ADHD-like behaviors in SHRs are not restricted to males (Sagvolden and Berger, 1996; Berger and Sagvolden, 1998). Whereas low doses of MPH are sufficient to improve behavioral deficits observed in ADHD patients, hyperactive behavior in SHR is ameliorated by high doses of *D*-amphetamine or MPH (Myers et al., 1982; Wultz et al., 1990). Recently, juvenile stroke-prone spontaneously hypertensive rats (SHRSP), a substrain of SHR, have been proposed as animal model of a developmental disorder more reminiscent of ADHD (Ueno et al., 2002). Importantly, only male SHRSP exhibit cognitive dysfunction and ADHD-like behavioral deficits, which are ameliorated by low MPH doses (Ueno et al., 2002). In addition, SHRSP, but not SHR, show significantly reduced regional cerebral blood flow (rCBF) in the frontal cortex (Jesmin et al., 2004a, b), as do ADHD patients (Lou et al., 1989; Yamori and Horie, 1977). These reports suggest that SHRSP represent a superior animal model of ADHD.

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) are essential for memory acquisition and learning (Fukunaga et al., 2009; Alkon et al., 2007). Indeed, activation of both kinases is critical to induce long-term potentiation (LTP) (Giese et al., 1998; Collingridge et al., 2004). Likewise, extracellular signal-regulated kinase (ERK) is associated with LTP maintenance via modulating cAMP-responsive element-binding protein (CREB)/brain derived neurotrophic factor (BDNF) signaling (Xia and Storm, 2005). In rodents such kinase activities in the PFC are critical for PFC-dependent behavior, including cognition (Dash et al., 2007; Yabuki et al., 2013; Wang et al., 2012). However, it is not known whether SHRSP exhibit altered neurochemical function.

Here, we investigated the neurochemical basis of ADHD-like behavior in SHRSP. We found that these animals exhibit aberrantly elevated CaMKII activity in the mPFC, but not in the hippocampal CA1 region, and showed upregulated expression of dopamine D2 receptor (D2R) expression in the mPFC.

2. Results

2.1. Acute MPH administration attenuates inattention and cognitive dysfunction in SHRSP.

Since acute intraperitoneal administration of MPH improves impaired alternation behavior in SHRSP (Ueno et al., 2002), we initially confirmed whether oral MPH administration improves inattention and cognitive impairment, as assessed by Y-maze and novel object recognition tasks, respectively, in SHRSP. In all treatment groups we observed a significant group effect of spontaneous alternation behavior [$F(3, 19) = 8.438$, $p < 0.01$]. Consistent with our previous observation (Ueno et al., 2002), SHRSP showed impaired alternation behavior and increased total arm entries compared with Wistar-Kyoto (WKY) rats, which served as controls (alternation behavior; WKY rats: $75.7 \pm 3.6\%$, SHRSP: $55.4 \pm 2.4\%$, $p < 0.01$ vs. WKY rats; total arm entries; WKY rats: $15.5 \pm 1.1\%$, SHRSP: 23.8 ± 1.5 , $p < 0.01$ vs. WKY rats; Fig 1A, B). Acute administration of MPH (at 1 mg/kg, p.o.) significantly improved impaired alternation behavior and antagonized the increase in total arm entries seen in SHRSP without affecting alternation behavior in WKY rats (alternation behavior; $68.5 \pm 4.2\%$, $p > 0.05$ vs. MPH-treated WKY rats, $p < 0.05$ vs. SHRSP; total arm entries; 9.3 ± 0.5 , $p > 0.05$ vs. MPH-treated WKY rats, $p < 0.01$ vs. SHRSP; Fig 1A, B).

To evaluate cognitive function we also assessed a novel object recognition task (Prickaerts et al., 2002). In a training session, we observed no difference in the discrimination index using the same object in all groups (Fig 1C, left). After a 24-hour retention interval between trial and test sessions, control WKY rats could discriminate novel from familiar objects (Fig 1C, right), while SHRSP could not (Fig 1C, right).

Under the same conditions, SHRSP administered MPH (at 1 mg/kg, p.o.) showed a significantly higher discrimination index for the novel object (Fig 1C, right). These findings indicate that oral MPH administration improves attention and antagonizes cognitive defects observed in SHRSP.

2.2. Acute MPH administration rescues hyperactivated CaMKII seen in the mPFC of SHRSP.

ADHD is associated with PFC hypofunction (Castellanos and Tannock, 2002), and SHRSP show reduced levels of neurotransmitters in that region, such as dopamine and glycine (Nakamura et al., 2001; Togashi et al., 2002). Thus, we investigated neuronal activity related to cognition to define mechanisms underlying cognitive dysfunction in SHRSP. Because CaMKII activity is crucial for successful performance of learning and memory tasks in rodents (Lisman et al., 2002; Fukunaga et al., 2009), we asked whether CaMKII activity is changed in the SHRSP mPFC. Ca^{2+} -independent CaMKII activity was significantly elevated in the SHRSP mPFC compared to WKY rats, without a change in total CaMKII levels. (Ca^{2+} -independent CaMKII activity; WKY rats: 22.9 ± 1.9 , SHRSP: 29.1 ± 2.4 , $p < 0.05$ vs. WKY rats; Fig 2A). We also observed aberrantly increased CaMKII activity in the SHRSP mPFC following analysis of CaMKII autophosphorylation levels (CaMKII α : $240.2 \pm 17.6\%$ of WKY rats, $p < 0.01$ vs. WKY rats; CaMKII β : $221.9 \pm 23.1\%$ of WKY rats, $p < 0.01$ vs. WKY rats; Fig 2B, C). By contrast, ERK1/2 and PKC α phosphorylation levels were unchanged in the mPFC of SHRSP compared with WKY control rats (Fig 2 B, D, E).

As acute MPH administration improves PFC-dependent behavioral deficits, including cognitive dysfunction (Fig 1), we speculated that MPH might attenuate

aberrantly elevated CaMKII autophosphorylation seen in SHRSP. As expected, levels of autophosphorylated-CaMKII in MPH-treated SHRSP were comparable to similarly treated WKY rats (Fig 2B, C). In addition, ERK1/2 and PKC α phosphorylation levels were unchanged between WKY rats and SHRSP with or without MPH treatments (Fig. 2 B, D, E).

2.3. Phosphorylation of CaMKII, ERK1/2 and PKC α is unchanged in the hippocampal CA1 region of SHRSP.

We next investigated kinase activities in CA1 of the hippocampus, a brain region important for acquisition of learning and memory. Unexpectedly, total and phosphorylated protein levels of CaMKII, ERK1/2 and PKC α were not significantly changed in the hippocampal CA1 region between WKY rats and SHRSP (Fig 3)

2.4. Upregulated GluR1 phosphorylation seen in SHRSP is ameliorated by MPH administration.

We confirmed synaptic effects of aberrantly elevated CaMKII activity by measuring phosphorylation of postsynaptic GluR1 (Ser 831) and presynaptic synapsin I (Ser 603), both CaMKII kinase substrates. As expected, GluR1 (Ser 831) phosphorylation markedly increased in the mPFC of SHRSP compared to WKY rats ($145.2 \pm 15.8\%$ of WKY rats, $p < 0.05$ vs. WKY rats; Fig. 4A, E). On the other hand, synapsin I (Ser 603) phosphorylation remained unchanged in the mPFC of SHRSP (Fig. 4A, B). In addition, GluR1 phosphorylation at Ser 845, a cAMP-dependent protein kinase (PKA) substrate, was unchanged in SHRSP (Fig 4A, E). These results suggest that postsynaptic CaMKII activity is upregulated in the mPFC of SHRSP. Total GluR1

and synapsin I protein levels were unchanged between WKY rats and SHRSP (Fig 4 A, B, C). As MPH administration attenuated aberrant CaMKII activity seen in SHRSP, MPH also antagonized upregulated-GluR1 (Ser 831) phosphorylation levels ($105.3 \pm 12.6\%$ of MPH-treated WKY rats, $p < 0.05$ vs. MPH-treated WKY rats; Fig. 4A, E).

2.5. Dopamine D2 receptor levels are upregulated in the mPFC of SHRSP

Finally, we investigated whether the dopaminergic system functions normally in the mPFC of SHRSP. Immunoblotting showed that levels of the dopamine D2 receptor (D2R), which is coupled with the G protein $G_{\alpha i}$ and inhibits adenylyl cyclase, were significantly increased in the mPFC of SHRSP compared with WKY rats ($185.4 \pm 29.6\%$ of WKY rats, $p < 0.05$ vs. WKY rats; Fig. 5A, B). MPH treatment, however, failed to restore increased D2R expression to control levels ($167.2 \pm 29\%$ of MPH-treated WKY rats, $p < 0.05$ vs. MPH-treated WKY rats; Fig. 5A, B). Levels of dopamine D1 receptor (D1R), which is coupled with $G_{\alpha s}$ and activates adenylyl cyclase, were unchanged in the mPFC of SHRSP (Fig. 5A, B).

3. Discussion

In present study, we demonstrated ADHD-like behavioral deficits in SHRSP and identified potential mechanisms underlying inattention and cognitive impairment seen in this animal model. We report two key observations. First, we observed aberrantly elevated CaMKII activity in postsynaptic regions of neurons in the SHRSP mPFC, an activity rescued by acute MPH administration. In addition, D2R levels were upregulated in the SHRSP mPFC.

Converging evidence from genetic and neuro-imaging studies supports a dopaminergic hypothesis of ADHD. Among several candidate genes, those encoding dopamine transporter (DAT) and receptor proteins are thought to contribute most significantly to ADHD pathogenesis (Cook et al., 1995; Gizer et al., 2009; Wu et al., 2012). A neuro-imaging study using positron emission tomography with [fluorine-18] fluorodopa revealed significantly low DOPA decarboxylase activity and reduced dopamine storage activities in the mPFC of ADHD patients compared with healthy subjects, suggesting that the dopaminergic system of ADHD patients is hypofunctional in vivo (Ernst et al., 1998). Here, we observed increased D2R expression in the mPFC of SHRSP. Such an increase may be a compensatory mechanism in response to low extracellular dopamine levels (Nakamura et al., 2001). Another potential mechanism underlying D2R upregulation could be increased CaMKII activity, as CaMKII can activate D2R transcription through NF- κ B signaling (Takeuchi et al., 2002). Presynaptic D2R is an autoreceptor regulating dopamine synthesis and release (Benoit-Marand et al., 2001; Dickinson et al., 1999; Rouge-Pont et al., 2002; Schmitz et al., 2002), suggesting that upregulated D2R could act to reduce dopamine content of mPFC neurons in SHRSP as a feedback mechanism. In addition, clinical meta-analysis has revealed a

high degree of D2R polymorphism in individuals with ADHD (Wu et al., 2012). Moreover, in animal models of ADHD, deletion of D2R or treatment with D2R antagonists alleviates behavioral deficits (Terranova et al., 2005; Fan et al., 2010). Thus, we suggest that elevated D2R expression levels possibly occurring in response to decreased dopamine levels in mPFC neurons are associated with ADHD-related behavior in SHRSP. Further studies using D2R antagonists are required to elucidate the functional relevance of the D2R upregulation.

Several reports suggest that elevated CaMKII activity is associated with disruption in synaptic plasticity and cognition. Transgenic mice with constitutively active CaMKII (T286D) show decreased excitatory postsynaptic currents (EPSCs) in the hippocampus (Pi et al., 2010). *Ube3a* maternal-deficient mice, which are models of Angelman Syndrome (AS), show contextual learning and LTP deficits with markedly increased CaMKII autophosphorylation levels in the hippocampus (Jiang et al., 1998; Weeber et al., 2003). Likewise, previously we found significantly increased CaMKII activity in the mPFC of α -thalassemia X-linked mental retardation (ATRX) mutant mice, which show mild learning impairments (Nogami et al., 2010; Shioda et al., 2011). Here, in the mPFC of SHRSP, we observed hyperactivated CaMKII without alterations in the activity of other Ca^{2+} -regulated protein kinases such as PKC. In addition, MPH rescued not only behavioral deficits but increased CaMKII activity, suggesting that inattention and cognitive impairment seen in SHRSP is due to aberrantly elevated CaMKII activity in the mPFC. However, mechanisms underlying elevated CaMKII activity in SHRSP remain unclear. The previous works defined that decreased protein phosphatase 1 (PP1) activity likely accounts in part for elevated CaMKII activity in ATRX mutant mice (Shioda et al., 2011) as well as in AS model mice (Weeber et al., 2003). In particular,

SHRSP exhibit similarities to ATRX mice, such as increased PKC, ERK and Ca^{2+} -independent CaMKII activity (Shioda et al., 2011). Importantly, allelic variations in the gene encoding protein phosphatase 2 regulatory subunit B gamma (PPP2R2C), a PP2A regulatory subunit, are reportedly associated with ADHD (Borsotto et al., 2007; Jacob et al., 2012). In addition, the gene encoding PPP2R2C is disrupted in a condition known as autosomal dominant intellectual disability, which is defined by impaired cognitive and adaptive behavior (Backx et al., 2010). Therefore, protein phosphatase activities may play a key role in the elevated CaMKII activity and behavioral deficits observed in SHRSP.

In the mPFC, MPH increases extracellular dopamine levels mainly via blocking norepinephrine transporters (NETs), as the density of DATs on dopaminergic neurons is very low (Sesack et al., 1998) and NETs account for dopamine reuptake in those cells (Carboni et al., 1990; Tanda et al., 1997; Morón et al., 2002). Dopamine stimulation by cocaine enhances formation of a heteroreceptor containing D2R and N-methyl-D-aspartate (NMDA) receptor NR2B subunits, resulting in disassociation of CaMKII from NR2B and inhibition of NMDA receptor-mediated currents (Liu et al., 2006). Moreover, postsynaptic D2R activation promotes PP1 activation by decreasing phosphorylation levels of dopamine- and cAMP-regulated phosphoprotein-32 at Thr-34 through decreased PKA activity and increased PP2B activity (Bateup et al., 2008; Nishi et al., 1997; Beaulieu and Gainetdinov, 2011). These observations suggest that MPH-stimulated increases in extracellular dopamine antagonize CaMKII activity by stimulating postsynaptic D2R signaling in the mPFC of SHRSP.

In conclusion, we find that oral MPH administration can improve inattention and cognitive impairment observed in SHRSP. ADHD-related behavioral deficits were

closely associated with aberrant CaMKII activity in the mPFC, but not in the hippocampal CA1 region. However, the mechanism underlying elevated activity of CaMKII remains unclear. Future studies should address this question by evaluating expression of genes encoding protein phosphatases as well as the activities of those encoded proteins. Overall, we propose that modulation of CaMKII activity represents an attractive potential therapy to improve behavioral impairment seen in ADHD patients.

4. Experimental procedures

4.1. Animals

Male SHRSP/Ezo rats and normotensive control WKY rats were obtained from Health Sciences University of Hokkaido, housed under conditions of constant temperature (23 ± 1 °C) and humidity ($55 \pm 5\%$) on a 12-h light-dark cycle (light; 9-21 h), and fed ad libitum. All animal procedures were approved by the Committee on Animal Experiments at both Tohoku University and Health Sciences University of Hokkaido. Efforts were made to minimize suffering and reduce the number of animals used. For experiments, juvenile (aged 6-7 weeks) male SHRSP/Ezo and WKY rats were used (Hiraide et al., 2013).

4.2. MPH treatment

Methylphenidate hydrochloride (Novartis Pharma, Tokyo, Japan) was dissolved in 0.5% sodium carboxymethyl cellulose. Animals were administered 1 mg/kg of MPH orally 1 hr prior to behavioral tests or decapitation prior to western analysis.

Three sets of experiments were conducted using different animals. (1) Group one was subjected to a Y-maze task (WKY rats: $n = 8$, SHRSP: $n = 8$; WKY rats treated with MPH: $n = 3$, SHRSP treated with MPH: $n = 4$). (2) Group two was subjected to a novel object recognition task (WKY rats: $n = 8$, SHRSP: $n = 8$; WKY rats treated with MPH: $n = 5$, SHRSP treated with MPH: $n = 5$). (3) Group three was used to collect tissue samples used for western analysis (WKY rats: $n = 5-6$, SHRSP: $n = 4-6$; WKY rats treated with MPH: $n = 4$, SHRSP treated with MPH: $n = 5$) and kinase assays CaMKII (WKY rats: $n = 8$, SHRSP: $n = 6$) after decapitation.

4.3. Y-maze task

Spontaneous alternation behavior in a Y-maze task, which requires attention and working memory (Katz and Schmalz, 1980; Sarter et al., 1988), was assessed as described (Yabuki and Fukunaga, 2013). The apparatus consisted of three identical arms ($50 \times 16 \times 32$ cm) made of black plexiglas. Animals were placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The sequence of arm entries was recorded manually. An alternation was defined as entries into all three arms on consecutive choices. The maximum number of alternations was defined as the total number of arms entered minus two, and the percentage of alternations was calculated as $\text{actual alternations} / \text{maximum alternations} \times 100$. The total number of arms entered during the session was also counted.

4.4. Novel object recognition task

The novel object recognition task was assessed as described (Yabuki et al., 2013). In brief, rats were individually habituated to an open-field box ($75 \times 75 \times 45$ cm) for two days. The experimenter scoring behavior was blind to the treatment. During the acquisition phase, two objects of the same material were placed symmetrically in the center of the chamber for 10 min. One hour later, the animals were allowed to explore the objects in the same manner, and training was repeated three times. Twenty-four hours later, one object was replaced by a novel object, and exploratory behavior was again analyzed for 10 min. After each session, objects were thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an object was defined as rearing on the object, sniffing it at a distance of less than 1 cm, or touching it with the

nose. Successful recognition was reflected by preferential exploration of the novel object. Discrimination of spatial novelty was assessed by comparing the difference between exploratory contacts of novel and familiar objects and the total number of contacts with both, making it possible to adjust for differences in total exploration contacts.

4.5. Kinase assay

After rats were decapitated, mPFC tissues were dissected, frozen in liquid nitrogen, and stored at -80°C . The tissues were homogenized in 40 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $\text{Na}_4\text{P}_2\text{O}_7$, 100 mM β -glycerophosphate, 25 mM NaF, 0.1 mM leupeptin, 75 μM pepstatin A, 0.05mg/ml aprotinin, and 1 mM dithiothreitol (homogenizing buffer). Insoluble material was removed by a 10 min centrifugation at 20,400 g from a tissue homogenate. Protein concentration was determined using Bradford's assay.

CaMKII activity was assessed as described (Fukunaga et al., 1993). The standard buffer contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 0.1 mM $[\text{Y-}^{32}\text{P}]$ ATP (3,000-5,000 cpm/pmol), 1 mg/ml bovine serum albumin, and 40 μM Syntide-2. To measure total CaMKII activity, the assay contained 1 mM CaCl_2 and 3 μM CaM, whereas to measure Ca^{2+} -independent activity, the assay contained 1 mM EGTA rather than CaCl_2 , and CaM. Reactions were initiated by addition of brain extract (1.5 μl). After 4 min incubation, 15 μl of the mixture was spotted on P-cellulose paper squares and processed as described (Roskoski, 1983).

4.6. Western blotting analysis

Western blotting analyses were carried out as described (Yabuki et al., 2013). In brief, after decapitation, tissue from mPFC or dorsal hippocampus CA1 region was dissected, frozen in liquid nitrogen, and stored at -80°C . For assays, frozen samples were homogenized in 200 μl buffer containing 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM Na_3VO_4 , 40 mM sodium pyrophosphate, 50 mM NaF, 100 nM calyculin A, 50 $\mu\text{g/ml}$ leupeptin, 25 $\mu\text{g/ml}$ pepstatin A, 50 $\mu\text{g/ml}$ trypsin inhibitor, and 1 mM dithiothreitol. Insoluble material was removed by a 10 min centrifugation at 20,400 g. Protein concentration was determined using Bradford's assay, samples were boiled 3 min in Laemmli's sample buffer, and equivalent amounts of protein were electrophoresed on SDS-polyacrylamide gels and transferred to an Immobilon polyvinylidene difluoride membrane for 2 h at 70 V. After blocking with TTBS solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% fat-free milk powder for 1 h at room temperature, membranes were incubated overnight at 4°C with anti-phospho-CaMKII (α/β -Thr286/287) (1:5000; Fukunaga et al., 1988), anti-CaMKII (1:5000; Fukunaga et al., 1988), anti-phospho-ERK (Thr202/Tyr204) (1:1000; Cell Signaling, Woburn, MA, USA), anti-ERK (1:1000; Cell Signaling), anti-phospho-PKC α (Ser-657) (1:1000; Millipore, Billerica, MA, USA), anti-PKC α (1:1000; Millipore), anti-phospho-GluR1 (Ser-831) (1:1000; Millipore), anti-GluR1 (1:1000; Millipore), anti-phospho-Synapsin I (Ser-603) (1:1000; Millipore), anti-Synapsin I (1:1000; Millipore), anti-dopamine D1 receptor (1:2500; Narushima et al., 2006), anti-dopamine D2 receptor (1:1000; Millipore), or anti- β -tubulin (1:10,000; Sigma-Aldrich, St Louis, MO, USA). After washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in TTBS solution for 60 min at room temperature. Blots

were developed using an ECL immunoblotting detection system (Amersham Biosciences) and visualized on X-ray film (Fuji Film, Tokyo, Japan). Autoradiographic films were scanned using densitometry (Lasergraphics, Irvine, CA, USA) and quantitated using Image Gauge version 3.41 (Fuji Film). We confirmed that the same amounts of protein are applied to SDS-PAGE by β -tubulin immunoblotting. The densities of proteins were normalized with densities of β -tubulin levels.

4.5. Statistical analysis

Results are expressed as means $\pm$ standard error of the mean (SEM). Significant differences were determined using Student's *t*-test for two-group comparisons. For analysis of spontaneous alternation behavior in a Y-maze task, a one-way ANOVA followed by a Fisher's PLSD test was used for multigroup comparisons. $p < 0.05$ represented a statistically significant difference.

Disclosure/conflict of interest

The authors have no conflict of interest.

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Figure Legends

Fig. 1 Inattention and cognitive impairment observed in SHRSP are improved by oral MPH administration.

(A) Alternation in a Y-maze task was measured in juvenile male SHRSP and WKY (control) rats. Alternation activities in untreated SHRSP significantly decreased compared with activity seen in WKY controls, an effect rescued by acute MPH administration (1.0 mg/kg, p.o.) (n = 3-8 per group). Error bars represent SEM. ** p < 0.01 vs. WKY rats. (B) In a Y-maze task, MPH administration rescued the increased number of total arm entries seen in SHRSP (n = 3-8 per group). Error bars represent SEM. ** p < 0.01 vs. WKY rats. (C) No differences between groups were seen in the trial session in the novel object recognition task (left; n = 5-8 per group). In the test session, SHRSP failed to discriminate novel from familiar objects, but MPH antagonized this effect (right; n = 5-8 per group). Error bars represent SEM. ** p < 0.01 vs. the familiar group.

Fig. 2 MPH administration attenuates elevated CaMKII activity observed in the mPFC of SHRSP.

(A) Increased Ca^{2+} -independent CaMKII activity was observed in the mPFC of SHRSP without a change in total CaMKII activity (n = 6-8 per group). Error bars represent SEM. * p < 0.05 vs. WKY rats. (B) Representative images of western blots probed with antibodies against autophosphorylated CaMKII (Thr-286), CaMKII, phosphorylated ERK (Thr-202/Tyr-204), ERK, phosphorylated PKC α (Ser-657) and PKC α in the mPFC. (C, D) Densitometric quantitation of CaMKII, ERK and PKC α as total and phosphorylated proteins. Data are expressed as a percent of the value seen in untreated

WKY rats (n = 4-6 per group). Error bars represent SEM. ** p < 0.01 vs. WKY rats.

Fig. 3 Ca²⁺-regulated kinase phosphorylation levels are unchanged in the hippocampal CA1 region.

(A) Representative images of western blots of tissues from the hippocampal CA1 region probed with antibodies against autophosphorylated CaMKII (α/β ; Thr-286/287), CaMKII, phosphorylated ERK (Thr-202/Tyr-204), ERK, phosphorylated PKC α (Ser-657) and PKC α (B, C, D) No differences in total or phosphorylated protein levels were seen. Data are expressed as a percent of values seen in WKY rats (n = 4-6 per group). Error bars represent SEM.

Fig. 4 Increased phosphorylation of GluR1, a CaMKII substrate, seen in the SHRSP mPFC is antagonized by MPH administration.

(A) Representative images of western blots of mPFC tissues probed with antibodies against phosphorylated Synapsin I (Thr-603), and Synapsin I, phosphorylated GluR1 (Ser-831, Ser-845) and GluR1. The protein and phosphorylation levels of synapsin I (Syn I) and GluR1 are summarized in the panel of B, C, and D. There were no differences in total and phosphorylated Synapsin I levels (n = 4-6 per group). Only GluR1 phosphorylation at Ser-831 was elevated in the mPFC, while total protein levels were unchanged. Oral MPH administration decreased phosphorylated GluR1 (Ser-831) immunoreactivity. Data are expressed as a percent of values seen in untreated WKY rats (n = 4-6 per group). Error bars represent SEM. * p < 0.05 vs. WKY rats.

Fig. 5 Dopamine D2 receptor protein levels increase in the mPFC of SHRSP.

(A) Representative images of western blots of mPFC tissues probed with antibodies against dopamine D2 and D1 receptors. (B) Densitometric quantitation of dopamine D2 and D1 receptors. Data are expressed as a percent of the value seen in untreated WKY rats (n = 4-6 per group). Error bars represent SEM. * $p < 0.05$ vs. WKY rats.

Fig. 1

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