Methylphenidate and Guanfacine Ameliorate ADHD-Like Phenotypes in *Fez1*-Deficient Mice

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Keywords

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Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder that, while prevalent, has a stagnant track record for advances in treatment. The limited availability of animal models with appropriate face and predictive validities has hampered progress in developing novel neurobiological hypotheses and testing new therapeutic options for this condition. Here, we report that mice deficient in *Fez1*, a gene specifically expressed in the nervous system with documented functions in neurodevelopment, show hyperactivity and impulsivity phenotypes, which are ameliorated by administering methylphenidate (MPH) or guanfacine (GFC), two pharmacological agents used for ADHD treatment. *Fez1*-knockout (KO) mice show reduced expression of tyrosine hydroxylase in the midbrain and the

brain stem and have reduced levels of dopamine, norepinephrine, or their metabolites in both the nucleus accumbens and the prefrontal cortex. These neurochemical changes in *Fez1*-KO mice were normalized by MPH or GFC. We propose that *Fez1*-KO mice can be used as a model to evaluate the role of altered neurodevelopment in the manifestation of ADHD-like behavioral phenotypes, as well as to investigate the neurobiological mechanisms of existing and new pharmacotherapeutic agents for ADHD.

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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental condition which afflicts 5–7% of children and around 4% of adults worldwide [1, 2]. Major symptoms of ADHD include inattention and/or impulsivity and hyperactivity [3], accompanied by

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varying degrees of cognitive, emotional, or motivational deficits, leading to major burdens at personal and socioeconomic levels [4–6]. Several clinical studies using positron emission tomography (PET) or functional magnetic resonance imaging have shown depressed dopamine (DA) activity in the caudate, nucleus accumbens (NAc), or limbic system in ADHD [7–9], whereas additional PET studies have shown that depressed DA activity is not specifically associated with ADHD but rather linked to low performance in an attention task demonstrated by both healthy individuals and those with ADHD [10]. At present, the etiological and pathophysiological mechanisms underlying this disorder remain unknown.

Methylphenidate (MPH), a psychostimulant that blocks the DA reuptake activity of the DA transporter (DAT), is a first-choice pharmacotherapy to treat this condition, despite the risk of developing addiction and psychosis with prolonged use or overdose [11–13]. More recent efforts to develop safer pharmacotherapies for ADHD have led to the approval of guanfacine (GFC), a selective agonist of the G_i protein-coupled α_{2A} adrenergic receptor, which inhibits cAMP (3',5'-cyclic adenosine monophosphate)-mediated signaling, leading to improved neurocognitive function [14]. However, a lack of appropriate animal models for ADHD has remained a roadblock for advancing our understanding of the pathophysiology of this condition, as well as for developing more effective treatment options.

Previous studies have led to several rodent models of ADHD-like symptoms, including spontaneously hypertensive rats (SHR) [15, 16], *DAT*-knockout (KO) mice [17, 18], and dopamine receptor 4 (*D4R*)-KO mice [19]. In addition, a recent study has established a hyperactive line of mice through phenotypic selection performed over multiple generations [20]. Nonetheless, there are limitations associated with each model; some of these models have only face validity with limited clinical relevance or atypical psychopharmacological measures. Most of these studies have shown little consideration of neurodevelopmental aspects of ADHD etiology.

The gene encoding FEZ1 (fasciculation and elongation protein zeta-1) is specifically expressed in the nervous system, with the highest levels of expression during neurodevelopment [21]. FEZ1 is involved in several neurodevelopmental processes, such as neurite extension [21, 22], dendritic arborization [23], axonal transport [24, 25], and neuronal migration [26]. In addition, we have previously reported that *Fez1*-KO mice have a hyperlocomotion phenotype [27]. In the current study, we report that *Fez1*-KO mice show additional ADHD-related behavior-

al deficits that are ameliorated by administering MPH or GFC. Furthermore, we evaluate catecholamine profiles and their responses to these pharmacological agents in *Fez1*-KO mice.

Methods

Animals

Fez1-KO mice [27] were maintained on the C57BL/6J genetic background for at least 10 generations. *Fez1*-KO mice and their wild-type (WT) littermate controls were used for analyses.

Behavioral Assays

Male mice were tested using a battery of behavioral assays at 6–7 weeks of age, as previously described [28, 29]. The personnel performing the tests were blinded to the genotype of the mice. For the pharmacological rescue experiments, MPH (Sigma; 0.5 mg/kg body weight) or GFC (Sigma; 0.1 mg/kg body weight) was dissolved in saline and intraperitoneally (i.p.) injected 30 min prior to behavioral assessment once (for acute treatment) or for 2 weeks (once daily for chronic treatment).

Locomotor Activities. Each mouse was placed at the center of an open field box ($W \times D \times H = 40 \times 40 \times 27$ cm, 50 lux), and locomotor activities were tracked over 60 min. Distance traveled (cm) was analyzed in 5-min bins using EthoVision (Noldus).

Elevated Plus Maze. Each mouse was placed at the center of a maze (38×7 cm arms, 50 cm above the floor, 50 lux) with the head facing an open arm. Total distance traveled and time spent in the open and closed arms were recorded during the 15-min session using EthoVision (Noldus).

Cliff Avoidance Reaction. Cliff avoidance reaction was evaluated as previously described [28]. Briefly, each mouse was habituated in a dark room (2.5 lux) for 30 min prior to the test, then placed on a round platform (an inverted acrylic cylinder with a diameter of 18 cm, 30 cm above the floor), and its behavior was video recorded for 30 min.

Quantification of Catecholamines and Their Metabolites

To measure levels of DA, norepinephrine (NE), and their metabolites, mice ($n = 5$ per group, male, 7 weeks old) were injected (i.p.) with either saline, MPH (0.5 mg/kg body weight), or GFC (0.1 mg/kg body weight) 30 min before decapitation, and the brains were quickly removed and sliced into 1-mm-thick sections using ice-cold Brain Matrices (Ted Pella). The NAc and the prefrontal cortex (PFC) were sampled using a punch-out device (i.d. 1.2 mm; Harris), homogenized with a sonicator for 30 s in 0.2 M perchloric acid buffer (2 mL per 100 mg tissue) containing 100 μ M EDTA and 100 μ g/mL isoproterenol as an internal standard, and kept on ice for 30 min. The homogenates were centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatants were collected, adjusted to a pH of 3 with 3 M CH_3COONa , and analyzed with a high-performance liquid chromatography system with an electrochemical detector (HTEC-500; Eicom, Kyoto, Japan). The column used for separation was an SC-5ODS (Eicom). The mobile phase was 0.1 M sodium phosphate buffer (0.1 M NaH_2PO_4 :0.1 M $\text{Na}_2\text{HPO}_4 = 1,000:160$, v/v), 1% methanol, 500 mg/L sodium sulfonate, and 50 mg/L EDTA.

Immunohistochemistry

Immunohistochemistry studies were performed as previously described [30]. For immunostaining of the midbrain, mice ($n = 4$ per group, male, 5–6 weeks old) were injected (i.p.) with either vehicle (saline), MPH (0.5 mg/kg body weight), or GFC (0.1 mg/kg body weight) daily for 14 days, and perfused transcardially with 4% paraformaldehyde at 7–8 weeks of age while under anesthesia (ketamine [100 mg/kg body weight]/xylazine [10 mg/kg body weight] mixture, i.p.). For immunostaining of the brain stem, mice ($n = 4$ per group, male) were similarly perfused at 7–8 weeks of age. The brains were then serially cut into 50- μ m-thick coronal sections using a vibratome (VT1200 S; Leica), and the sections from one cohort of mice (littermates of WT and *Fez1*-KO mice treated with each drug or vehicle) were simultaneously immunostained at 4 °C for 16 h with an anti-tyrosine hydroxylase (TH) antibody (mouse, 1:3,000; Abcam), followed by Alexa Fluor® 488-conjugated secondary antibody (Gibco) at room temperature for 1 h, and mounted using ProLong Gold antifade reagent.

Images of one optical section (1 μ m thick) were acquired via confocal microscopy (SP8, Leica; 20 $\times$ objective lens, NA = 0.75) from sections containing the ventral tegmental area and substantia nigra pars compacta, and from sections containing the locus coeruleus. In each mouse, 3–4 serial sections were analyzed. The levels of TH immunostaining were measured from each soma using ImageJ (NIH), with background levels in adjacent regions being subtracted, and the relative fluorescence intensity per soma was calculated by normalizing it by the average fluorescence intensity in WT mice, and analyzed across all sections from every mouse used.

Statistical Analysis

Behavioral assay data (means $\pm$ standard error of the mean [SEM]) were analyzed using one-way or two-way ANOVA (analysis of variance) followed by Bonferroni post hoc tests. Catecholamine measurements and immunohistochemistry data were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test, or by Mann-Whitney test (two-tailed). Statistical significance was marked the following way: * $p < 0.05$, ** $p < 0.01$.

Results

Hyperlocomotion Is Ameliorated by MPH or GFC in Fez1-KO Mice

To assess baseline activities of *Fez1*-KO mice, we used an open field box to track their locomotor behavior for 1 h, an experimental scheme widely used for testing ADHD-like hyperactivity in rodents [31]. The *Fez1*-KO mice displayed significantly higher locomotor activities than the WT littermate controls throughout the testing period (Fig. 1a), showing an approximately 30% increase in the total distance traveled (Fig. 1b), consistent with the initial characterization of this mouse line [27]. We next tested whether this hyperlocomotion phenotype was relevant to ADHD by testing the response of *Fez1*-KO mice to MPH or GFC, two pharmacotherapeutic agents used for ADHD treatment [13, 14]. The hyperlocomotion phenotypes in the *Fez1*-KO

mice were ameliorated by acute treatment with MPH (Fig. 1a, b) or GFC (Fig. 1c, d) ($F(55, 418) = 2.531$, $p < 0.0001$, for locomotion in 5-min bins [Fig. 1a, c]; $F(5, 38) = 4.511$, $p = 0.0025$, for total distance traveled [Fig. 1b, d]), as well as after chronic treatment with these agents (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000488081 for all online suppl. material). Note that the locomotor phenotypes in the control mice were not significantly affected by these treatments. The data suggested that *Fez1*-KO mice had psychopharmacological changes that might be shared with ADHD.

Impulsivity Is Ameliorated by MPH or GFC in Fez1-KO Mice

We next addressed whether *Fez1*-KO mice showed elevated impulsivity, another core symptom observed in ADHD. The elevated plus maze (EPM) has been used for evaluating not only emotionality but also impulsivity in several rodent models of ADHD [16, 32]. Consistent with the findings from the open field test, the *Fez1*-KO mice showed increased trends of locomotor activities ($F(5, 44) = 2.818$, $p = 0.0272$; Fig. 2a) and a significantly increased time spent in the open arms of the EPM ($F(5, 44) = 5.404$, $p = 0.0006$; Fig. 2b), suggesting that the *Fez1*-KO mice were less anxious or had an impulsive tendency when compared to the controls. These behavioral changes were normalized by acute treatment with GFC, but only insignificantly ameliorated with MPH (Fig. 2a, b). Note that these treatments did not affect behaviors in the controls.

To directly test impulsivity, *Fez1*-KO mice were assessed for cliff avoidance reaction on an elevated platform. Whereas the control mice showed high levels of cliff avoidance (i.e., low rates of dropping and jumping off the platform), the *Fez1*-KO mice showed progressively higher rates of dropping and jumping during the observation period ($F(58, 1,218) = 2.302$, $p < 0.0001$; Fig. 2c), suggesting that they showed more impulsive behaviors. We next tested whether GFC or MPH could rescue this phenotype. The lower rates of cliff avoidance reaction observed in the *Fez1*-KO mice were normalized by acute treatment with GFC (Fig. 2c, d). When MPH was acutely administered, the phenotype of the *Fez1*-KO mice was not completely normalized; however, we observed a trend for improvement, where MPH-treated *Fez1*-KO mice had cliff avoidance reactions higher than those of vehicle-treated KO mice ($p = 0.1957$) and lower than those of WT controls ($p = 0.0682$), showing a score in between (online suppl. Fig. 2). The data demonstrated that the impulsivity-like behaviors in *Fez1*-KO mice were insignificantly improved by MPH to a level comparable to that of the controls, sug-

Fig. 1. Hyperlocomotion is ameliorated by methylphenidate (MPH) or guanfacine (GFC) in *Fez1*-knockout (KO) mice. Locomotor activities in the open field box were evaluated for wild-type (WT) control mice treated acutely with saline ($n = 10$), MPH (0.5 mg/kg body weight) ($n = 5$), and GFC (0.1 mg/kg body weight) ($n = 5$), and for *Fez1*-KO mice treated with saline ($n = 8$), MPH ($n = 8$), and GFC ($n = 8$) (male, 6–7 weeks old). **a** Locomotor activities shown in 5-min bins for WT mice treated with saline or MPH, and *Fez1*-KO mice treated with saline or MPH. Statistical significance: $F(55, 418) = 2.531$, $p < 0.0001$ (two-way ANOVA with repeated measures); * $p < 0.05$ (Bonferroni post hoc test). **b** Total

distance traveled (cm) over the 60-min test period for the mice used in **a**. Statistical significance: $F(5, 38) = 4.511$, $p = 0.0025$ (one-way ANOVA); * $p < 0.05$ (Bonferroni post hoc test). **c** Locomotor activities shown in 5-min bins for WT mice treated with saline or GFC, and *Fez1*-KO mice treated with saline or GFC. Statistical significance: $F(55, 418) = 2.531$, $p < 0.0001$ (two-way ANOVA with repeated measures); * $p < 0.05$, ** $p < 0.01$. **d** Total distance traveled (cm) over the 60-min test period for the mice used in **c**. Statistical significance: $F(5, 38) = 4.511$, $p = 0.0025$ (one-way ANOVA); * $p < 0.05$, ** $p < 0.01$.

Fig. 2. Impulsivity is ameliorated by guanfacine (GFC) in *Fez1*-knockout (KO) mice. **a, b** The elevated plus maze was used to test wild-type (WT) control mice treated acutely with saline ($n = 11$), methylphenidate (MPH) (0.5 mg/kg body weight) ($n = 7$), and GFC (0.1 mg/kg body weight) ($n = 6$), and *Fez1*-KO mice treated with saline ($n = 8$), MPH ($n = 10$), and GFC ($n = 8$). Statistical significance: total distance traveled (cm) (**a**), $F(5, 44) = 2.818$, $p = 0.0272$; time spent in open arms (%) (**b**), $F(5, 44) = 5.404$, $p = 0.0006$ (one-way ANOVA); * $p < 0.05$, ** $p < 0.01$ (Bonferroni post

hoc test). **c** Cliff avoidance behaviors were scored for 30 min for WT mice treated acutely with saline ($n = 16$), *Fez1*-KO mice treated with saline ($n = 16$), and *Fez1*-KO mice treated with GFC ($n = 13$). The percentage of mice that stayed on the platform during the test period was plotted. Statistical significance: $F(58, 1,218) = 2.302$, $p < 0.0001$ (two-way ANOVA with repeated measures); ** $p < 0.01$. **d** Percentage of cliff avoidance reaction (CAR) at the end of the test. Statistical significance: $F(2, 42) = 4.489$, $p = 0.0171$ (one-way ANOVA); * $p < 0.05$.

gesting that MPH shows a partial rescuing effect against the impulsivity-like behaviors of *Fez1*-KO mice, in agreement with the results obtained with the EPM (Fig. 2a, b). These data imply that *Fez1*-KO mice may provide a model for elevated impulsivity that might be relevant to a core behavioral change observed in ADHD.

Reduced Levels of Catecholamines in *Fez1*-KO Mice Are Rescued by MPH or GFC

As a first step to address psychopharmacological changes in *Fez1*-KO brains that might underlie ADHD-like behaviors, we assessed the levels of DA and its metabolites in the NAc, a major target of dopaminergic ef-

Fig. 3. Profiles of catecholamines and their metabolites in the nucleus accumbens (NAc) of *Fez1*-knockout (KO) mice. Levels of catecholamines and their metabolites in the NAc were measured by high-performance liquid chromatography using wild-type (WT) mice injected acutely with saline ($n = 5$), *Fez1*-KO mice treated with saline ($n = 5$), *Fez1*-KO mice treated with methylphenidate (MPH) (0.5 mg/kg body weight) ($n = 5$), and *Fez1*-KO mice

treated with guanfacine (GFC) (0.1 mg/kg body weight) ($n = 5$) (male, 7 weeks old). **a–c** Levels of dopamine (DA) (**a**), DOPAC (**b**), and homovanillic acid (HVA) (**c**) measured in the NAc. **d–f** Levels of norepinephrine (NE) (**d**), normetanephrine (NM) (**e**), and HMPG (**f**) measured in the NAc. Statistical significance: * $p < 0.05$, ** $p < 0.01$ (Kruskal-Wallis test followed by Dunn's multiple comparison test).

ferent fibers from the midbrain. Clinical studies using PET have previously reported that ADHD brains show disrupted DA neurotransmission in the NAc [7, 9, 33]. The *Fez1*-KO mice had significantly lower levels of DA in the NAc, which were normalized by acute administration of MPH or GFC ($p = 0.012$ by Kruskal-Wallis test) (Fig. 3a). Consistently, levels of DA metabolites (i.e., DOPAC [3,4-dihydroxyphenylacetic acid] and homova-

nillic acid) were also lower in the NAc of *Fez1*-KO mice, and were augmented by these agents ($p = 0.0118$ for DOPAC; $p = 0.0157$ for homovanillic acid; by Kruskal-Wallis test) (Fig. 3b, c). We also assessed the levels of NE and its metabolites, as GFC primarily augments NE activity [14]. The levels of NE in the NAc of *Fez1*-KO mice were significantly lower than those in WT mice, and they were normalized by acute administration of MPH or GFC

Fig. 4. Reduced expression of tyrosine hydroxylase (TH) in the midbrain is augmented by methylphenidate (MPH) or guanfacine (GFC) in *Fez1*-knockout (KO) mice. **a** Expression of TH in the ventral tegmental area and substantia nigra pars compacta in wild-type (WT) and *Fez1*-KO mice treated with saline, *Fez1*-KO mice treated with MPH (0.5 mg/kg body weight), and *Fez1*-KO mice treated with GFC (0.1 mg/kg body weight) (daily i.p. for 2 weeks, 7–8 weeks old when perfused). Scale bar, 200 μ m. **b** Fluorescence intensities of anti-TH immunostaining per soma (80–100 somas scored per section, 3–4 serial sections analyzed per animal; $n = 4$ animals used per group) above the background level were quantified by ImageJ, and normalized by the average fluorescence intensity in WT mice within each cohort (#1, #2, #3, and #4). Relative fluorescence intensities per soma from a representative section are plotted in the graph, and the overall average fluorescence intensities ($\pm$ SEM) across multiple sections per group are shown in red in the graph. Statistical significance: ** $p < 0.01$ (Kruskal-Wallis test followed by Dunn's multiple comparison test).

($p = 0.0115$ by Kruskal-Wallis test) (Fig. 3d). By contrast, the levels of its metabolites (i.e., normetanephrine and HMPG [4-hydroxy-3-methoxyphenylglycol]) were comparable between WT and *Fez1*-KO mice and were not significantly affected by acute administration of MPH or GFC ($p = 0.8095$ for normetanephrine; $p = 0.1224$ for HMPG; by Kruskal-Wallis test) (Fig. 3e, f).

We next assessed the levels of DA, NE, and their metabolites in the PFC, another target of dopaminergic efferent fibers from the midbrain and of noradrenergic efferent fibers from the locus coeruleus in the brain stem. The *Fez1*-KO mice had significantly lower levels of DA in the PFC, which were normalized by acute administration of MPH or GFC ($p = 0.0051$ by Kruskal-Wallis test) (online suppl. Fig. 3a). The levels of DA metabolites showed

similar trends consistent with the DA levels (online suppl. Fig. 3b, c). In addition, the levels of NE in the PFC of *Fez1*-KO mice were significantly lower than those in WT mice and were normalized by acute administration of MPH or GFC ($p = 0.0038$ by Kruskal-Wallis test) (online suppl. Fig. 3d), while the levels of NE metabolites were comparable between WT and *Fez1*-KO mice regardless of drug administration (online suppl. Fig. 3e, f). These data together suggested that *Fez1*-KO mice had altered catecholamine states that might be relevant to ADHD.

TH Expression Is Reduced in the Midbrain and the Brain Stem of Fez1-KO Mice

To investigate a biological basis for reduced catecholamine levels in *Fez1*-KO mice, we studied the expression

levels of TH, a key enzyme essential for catecholamine synthesis [34], via immunohistochemistry. The levels of TH immunoreactivity in neuronal somas in the midbrain of *Fez1*-KO mice were significantly lower than those of controls (Fig. 4a, b). Moreover, *Fez1*-KO mice chronically administered MPH or GFC had TH levels significantly greater than those in *Fez1*-KO mice treated with vehicle alone (1.000 ± 0.021 for WT mice with saline, 0.596 ± 0.014 for *Fez1*-KO mice with saline, 1.064 ± 0.025 for *Fez1*-KO mice with MPH, and 0.886 ± 0.017 for *Fez1*-KO mice with GFC; $p < 0.01$ by Kruskal-Wallis test) (Fig. 4a, b). In addition, the levels of TH immunoreactivity in the locus coeruleus in the brain stem of *Fez1*-KO mice were significantly lower than those of controls (1.000 ± 0.036 for WT mice, 0.650 ± 0.024 for *Fez1*-KO mice; $p < 0.01$ by Mann-Whitney test) (online suppl. Fig. 4). These data suggested that the reduced catecholamine levels in *Fez1*-KO mice could in part be due to lower expression of TH in the midbrain and the brain stem.

Discussion

In the present study, *Fez1*-KO mice showed hyperlocomotion and higher impulsivity-like phenotypes and had reduced levels of catecholamines in the NAc and the PFC along with consistent immunohistochemical changes in the midbrain and the brain stem. In addition, these changes could be ameliorated by MPH or GFC, two major pharmacotherapeutic agents used for ADHD treatment. An initial characterization of *Fez1*-KO mice also showed no apparent deficit in prepulse inhibition [27], a measure of sensorimotor gating. Collectively, these phenotypic and psychopharmacological alterations suggest that *Fez1*-KO mice may serve as a new model for ADHD-like symptoms with face and predictive validity. It remains to be tested whether this mouse model also has inattention phenotypes, additional diagnostic criteria for ADHD per the DSM-5, and whether those could be normalized by MPH or GFC. At present, we do not know the reason for the partial rescue of the impulsivity phenotype of *Fez1*-KO mice by MPH. Administering a higher dose of MPH or for a longer period of time may be necessary to achieve complete rescue, as has been tested in the clinic to determine the optimal dosing and duration of MPH for treating ADHD [35]. Nonetheless, the current data are consistent with clinical evidence that MPH is effective in alleviating both hyperactivity and impulsivity in ADHD.

Because we observed equivalent extents of significant rescue of hyperactivity in *Fez1*-KO mice by both acute and chronic treatments with MPH or GFC, as well as consistent increases in catecholamine contents via acute treatment, we anticipate seeing similar neurochemical changes after chronic treatment. Therefore, the behavioral rescue effect during chronic treatment could possibly be mediated by every single dose of drug treatment. Alternatively, this rescue effect via chronic treatment may be sustained by the recovery of TH expression achieved with the chronic treatment.

In our previous study, *Fez1*-KO mice showed higher sensitivity to MK-801, an *N*-methyl-D-aspartate receptor antagonist that elicits high levels of DA release in the NAc. Upon MK-801 injection, *Fez1*-KO mice showed significantly greater DA release in the NAc than control mice in microdialysis experiments [27]. Taken together with our current results, this mouse model is likely to have dysregulated catecholamine neurotransmission, which could further provide a psychopharmacological basis for the ADHD-like behavioral phenotypes observed.

Because MPH primarily blocks the activity of DAT, which is highly expressed in the caudate, NAc, and the limbic system, the primary sites of action of MPH are likely in these areas of the brain. However, the pharmacotherapeutic action of MPH could also be mediated through inhibition of the NE transporter in the PFC, leading to enhanced extracellular levels of NE and DA in the PFC and hence cognitive improvement and suppression of hyperactivity, as has been proposed to explain the therapeutic effect of MPH in *DAT*-KO mice [36]. By contrast, GFC primarily activates α_{2A} adrenergic receptor to strengthen NE neurotransmission in the PFC, which enhances cognitive function and suppresses impulsive/hyperactive behaviors in ADHD [37]. The mechanism by which GFC augmented DA levels in the NAc of *Fez1*-KO mice in the present study is currently unknown; it is possible that GFC indirectly regulates subcortical DA levels through the corticolimbic circuitry. For example, FEZ1 plays a role in anterograde transport of synaptic vesicles [24, 25], and could possibly regulate trafficking of DA-containing synaptic vesicles originating from the dopaminergic neurons in the midbrain, thereby controlling DA release in the NAc, as previously shown [27]. It remains to be studied whether GFC could augment mesolimbic DA trafficking via activation of the corticolimbic circuitry. In this regard, it should be noted that functional and structural abnormalities in the midbrain and the NAc (e.g., altered DAT levels, size of the substantia nigra)

have previously been reported in ADHD via clinical imaging studies [9, 33, 38, 39], as well as in a rodent model (i.e., SHR) [40].

Previous studies have provided a series of rodent models of ADHD-like symptoms, including SHR [15, 16], *DAT*-KO mice [17, 18], and *D4R*-KO mice [19], as well as a hyperactive line of mice established through phenotypic selection over multiple generations [20]. Although all these models share a hyperlocomotion phenotype, there are limitations associated with each model in terms of relevance to ADHD. For example, the SHR model was originally established as a hypertension model, and it has been difficult to determine whether the hyperactive phenotype is a primary deficit or a condition secondary to the hypertension phenotype. In addition, there are no appropriate parental control lines for SHR, making them hard to use for contemporary biomedical approaches toward drug development or pathophysiological studies [15]. *DAT*-KO mice show both face and predictive validity; however, several phenotypes, including high levels of DA (around 10 times that of controls) in the NAc and the presence of prepulse inhibition deficits, do not seem to be frequently observed in ADHD [31, 41]. *D4R* is one of the promising risk genes identified through genome-wide association studies for ADHD, and *D4R*-KO mice show hyperactivity; however, this line of mice shows atypical responses to pharmacological interventions commonly used for ADHD treatment [19]. Finally, while the hyperactive line of mice developed through phenotypic selection appears to reflect the complex nature of ADHD genetics, where alteration in no single gene is attributable to the hyperactive phenotype, it makes the downstream analysis of etiology and underlying pathophysiology more difficult [20].

Although the present study has demonstrated face and predictive validity for *Fez1*-KO mice as a model for ADHD-like symptoms, there is currently no direct genetic evidence linking *Fez1* to ADHD, except that *Fez1* is mapped in proximity of an ADHD “hot spot” region in chromosome 11 [42]. Besides, it is unlikely that a single genetic alteration causes the range of symptoms associated with ADHD. Nonetheless, to the best of our knowledge, *Fez1*-KO mice represent the first model of ADHD-like symptoms with a potential disturbance in neurodevelopment. Previous cell biology studies have shown that FEZ1 is involved in multiple aspects of neurodevelopment, including neurite elongation [21, 22], dendritic arborization [23], axonal transport [24, 25], and neuronal migration [26], by interacting with a number of proteins with documented neurobiological functions, including

kinesin-1 and DISC1 (disrupted in schizophrenia-1) [24–26]. Although our initial characterization of *Fez1*-KO mice revealed no gross anatomical changes, presumably due in part to a compensatory pathway through a family gene (*Fez2*) [27], we predict that either a microscopic deficit in the neuronal cytoarchitecture or a delay in neuronal development exists in *Fez1*-KO mice. We therefore posit that further analyses of *Fez1*-KO mice will facilitate dimensional studies on how disturbances in neurodevelopment may contribute to susceptibility to developing ADHD-like behavioral and psychopharmacological deficits.

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Statement of Ethics

Animal handling was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the Animal Research Committee at Kyoto University.

Disclosure Statement

We have no conflict of interest to declare.

Author Contributions

A. Sumitomo and T. Tomoda designed the study; A. Sumitomo and A. Saka conducted the experiments and statistical analysis; K. Ueta and K. Horike assisted in the behavioral experiments; K. Hirai conducted the histochemistry studies; N.J. Gamo, T. Hikida, T. Sakurai, and K.I. Nakayama provided the resources and analytical tools; N.J. Gamo edited the manuscript; A. Sawa and T. Sakurai provided advice and interpreted the data; and T. Tomoda guided the research and wrote the manuscript.

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