Dopaminergic loss of cyclin-dependent kinase-like 5 recapitulates methylphenidate-remediable hyperlocomotion in mouse model of CDKL5 deficiency disorder

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ABSTRACT

Cyclin-dependent kinase-like 5 (CDKL5), a serine-threonine kinase encoded by an X-linked gene, is highly expressed in mammalian forebrain. Mutations in this gene cause CDKL5 deficiency disorder, a neurodevelopmental encephalopathy characterized by early-onset seizures, motor dysfunction and intellectual disability. We previously found that mice lacking CDKL5 exhibit hyperlocomotion and increased impulsivity, resembling the core symptoms in attention-deficit hyperactivity disorder (ADHD). Here, we report the potential neural mechanisms and treatment for hyperlocomotion induced by CDKL5 deficiency. Our results showed that loss of CDKL5 decreases the proportion of phosphorylated dopamine transporter (DAT) in the rostral striatum, leading to increased level of extracellular dopamine and hyperlocomotion. Administration of methylphenidate (MPH), a DAT inhibitor clinically effective to improve symptoms in ADHD, significantly alleviated the hyperlocomotion phenotype in *Cdkl5* null mice. In addition, the behavioral effects of MPH were accompanied by a region-specific restoration of phosphorylated dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa, a key signaling protein for striatal motor output. Finally, mice carrying *Cdkl5* deletion selectively in DAT-expressing dopaminergic neurons, but not dopamine receptive neurons, recapitulated hyperlocomotion phenotype found in *Cdkl5* null mice. Our findings suggest that CDKL5 is essential to control locomotor behavior by regulating region-specific dopamine content and phosphorylation of dopamine signaling proteins in the striatum. The direct, as well as indirect, target proteins regulated by CDKL5 may play a key role in movement control and the therapeutic development for hyperactivity disorders.

INTRODUCTION

Cyclin-dependent kinase-like 5 (CDKL5, OMIM 300203) is a serine-threonine kinase encoded by the X-linked *CDKL5* gene (1). CDKL5 protein is highly expressed in forebrain neurons with prominent functions in neuronal morphogenesis, including dendritic arborization, spine stabilization and synaptic formation, as well as maintaining neural circuits (2-8). Mutations in *CDKL5* gene cause CDKL5 deficiency disorder (CDD, OMIM 300672) characterized by early-onset seizures starting at a few weeks of life, accompanied by autistic features, motor dysfunction and intellectual disability (9-13).

The first mouse model for CDD was developed by deletion of exon 6, resulting in a premature stop codon and loss of CDKL5 function (14). Alternative models were established later on with deletion of exon 4 or exon 2, respectively, on *Cdkl5* gene (15, 16). These CDKL5 deficiency mice exhibit impaired social interaction and motor coordination, as well as learning and memory deficits, resembling numerous CDD-related symptoms (14-17), thus fulfilling the construct and face validity of an animal model for CDD (18). In addition to the behavioral abnormalities, loss of CDKL5 also alters kinome profiles and subcellular localization of glutamate receptors (14, 19, 20), suggesting that CDKL5 may play a role in regulating multiple signal transduction pathways. Despite having discovered numerous substrates or interacting partners of CDKL5, such as amphyphysin 1, PSD95, EB2 and MAP1S (21-23), the causal and associative relationships between CDKL5-mediated signaling and behavioral phenotypes remain unclear.

We previously demonstrated that mice lacking CDKL5 exhibit autism-like phenotypes, such as impaired communication and sociability. These mice also develop

hyperlocomotion, resembling core symptoms of attention-deficit hyperactivity disorder (ADHD, OMIM 143465). In addition, their dopamine content is selectively elevated in the anterior forebrain, including the medial prefrontal cortex and the rostral part of the striatum (ST-r), compared to wild-type (WT) mice (17). The striatum is the main input nucleus of the basal ganglia that is known to control psychomotor behaviors and implicated in motor deficits of neurodevelopmental disorders (24-26). Unlike the caudal striatum (ST-c), the ST-r in rodents is mainly composed of striatonigral neurons distributed in the striosomal compartment, corresponding to the caudate nucleus in humans (27, 28). More than 98% of the striatal neurons are dopamine-receptive GABAergic projection neurons, composed of dopamine D1 receptor (D1R)-expressing "direct" pathway and D2R-expressing "indirect" pathways, oppositely tuning the excitability of motor thalamus and execute cortical motor commands (29-31). It is unclear if deregulation of dopamine signaling in striatal neurons accounts for hyperlocomotion in *Cdkl5* null mice.

As a striatum-enriched signaling protein, dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa (DARPP32, also known as phosphoprotein phosphatase-1 regulatory subunit 1B, PPP1R1B) functions as an inhibitor of protein phosphatase-1 (PP1) and an integrator of dopamine and glutamate signaling (32-34). DARPP32 is not only implicated in movement control and motivational behaviors, but also associated with numerous psychiatric disorders (35, 36). Given that loss of CDKL5 alters dopamine contents and enhances locomotor activity, it is possible that the expression or functional state of DARPP32 is affected by CDKL5 deficiency. Given the role of CDKL5 in regulating signaling pathways, the extent to which phosphorylation of DARPP32 would be affected by CDKL5 deficiency remains to be determined. In addition to DARPP32,

dopamine transporter (DAT) also plays a critical role in regulating synaptic dopamine levels and movement control. The aberrant expression of DAT has been implicated in pathogenesis of ADHD (37). Clinically, methylphenidate (MPH), a DAT inhibitor, has been extensively used as the first-line medication for ADHD (38). Moreover, phosphorylation of DAT protein at threonine 53 (pDAT) enhances the efficacy of dopamine reuptake and efflux leading to alteration of extracellular level of dopamine (39). It is possible that CDKL5 deficiency deregulates expression or phosphorylation of DAT, compromises dopamine reuptake and enhances postsynaptic dopamine signaling, leading to hyperactive locomotion.

To test this hypothesis, we measured the levels of extracellular dopamine, protein expression and phosphorylation of DAT and DARPP32 in the striatum, as well as the effects of MPH on locomotion in CDKL5 deficiency mice. To elucidate the role of CDKL5 in different neuronal types in the striatum, we also investigated the effects of conditional knockout (cKO) of CDKL5 in the present study.

RESULTS

Increased levels of extracellular dopamine in the ST-r of hyperactive *Cdkl5*^{-/y} mice

Our previous studies demonstrated that mice lacking CDKL5 exhibit hyperlocomotion mimicking clinical symptoms of ADHD (17). Accompanied with enhanced locomotor activity, these mice also display increased dopamine content in the ST-r while reduced dopamine level in the ST-c. To further elucidate whether the dopamine alterations measured from the tissue lysate reflects the synaptic level of dopamine, we measured the extracellular dopamine level in mouse striatum using *in vivo* microdialysis.

We collected cerebrospinal fluid through a microdialysis probe targeted to the ST-r of mice freely moving in an open field arena. Dopamine level was analyzed immediately with high-performance liquid chromatography (HPLC) (Fig 1). Right after the start of infusion of Ringer's solution, we assessed locomotion of the tested mice in the plastic "bowl" for 15 min in parallel with collection of dialysate. We found that CDKL5 deficient mice (KO, $Cdkl5^{-/y}$) exhibited enhanced locomotor activity compared to WT littermate controls (3861.9 ± 108.3 in KO vs. 2995.0 ± 92.2 in WT, $p < 0.01$)(Fig 2A, A', C), consistent with our previous observation using a square arena (17). In addition to behavioral hyperactivity, we found that the levels of dopamine released in the first collection of dialysate was elevated in $Cdkl5^{-/y}$ mice (24.4 ± 4.7 in KO vs. 11.7 ± 3.1 in WT, $p < 0.05$)(Fig 2B, B', D), suggesting that hyperlocomotion in mice lacking CDKL5 was tightly correlated with increased synaptic dopamine in the ST-r. We also measured the released dopamine levels from the ST-c of an independent cohort of $Cdkl5^{-/y}$ and control mice, and no difference was found between genotypes in the first dialysate (1.54 ± 0.43 in KO vs. 1.25 ± 0.64 in WT, $p = 0.5802$, n = 4). Notably, the extracellular dopamine levels and locomotor activity became comparable between genotypes after mice receiving subcutaneous saline administration ($p > 0.05$; Fig 2E, F). It is likely that continuous infusion of Ringer's solution "cleaned up" synaptic dopamine in the ST-r so that locomotion activity became equivalent in all mice. Our findings confirm that increased synaptic dopamine locally at the ST-r is critical for the hyperlocomotion phenotype in $Cdkl5^{-/y}$ mice.

Altered expression and phosphorylation of dopamine transporter in *Cdkl5⁻ᐟʸ* mice

Given that extracellular dopamine tone is determined by the function of DAT (40), and DAT is a well-documented therapeutic target for ADHD (41), it is likely that the increased level of synaptic dopamine in the ST-r in *Cdkl5⁻ᐟʸ* mice results from decreased reuptake of dopamine. Regarding that reuptake function of DAT was enhanced by kinase-mediated phosphorylation at threonine 53 (pDAT)(39, 42), we thus examined the protein levels of pDAT and total DAT in *Cdkl5⁻ᐟʸ* mice using immunoblotting.

We found that the protein level of DAT is increased in the ST-r (1.12 ± 0.22 in KO vs. 0.44 ± 0.08 in WT, $p < 0.05$), but reduced in the ST-c (0.46 ± 0.08 in KO vs. 1.16 ± 0.13 in WT, $p < 0.01$) in *Cdkl5⁻ᐟʸ* mice (n = 6 pairs, Fig. 3A, A', B). However, the levels of pDAT were similar in both the ST-r and ST-c in *Cdkl5⁻ᐟʸ* mice compared to WT littermate controls (Fig. 3A, A'). The proportion of DAT phosphorylation (i.e. the ratio of pDAT/DAT), a valid functional index reflects the DAT activity, was thus significantly reduced in the ST-r (0.99 ± 0.11 in KO vs. 2.07 ± 0.11 in WT, $p < 0.001$), but increased in the ST-c (1.86 ± 0.29 in KO vs. 0.70 ± 0.12 in WT, $p < 0.01$) in *Cdkl5⁻ᐟʸ* mice (Fig. 3C), suggesting that loss of CDKL5 may alter the efficacy of dopamine reuptake in a striatal subregion-specific manner. The reduced proportion of pDAT in the ST-r may decrease dopamine reuptake, contributing to increased synaptic dopamine levels and hyperlocomotion in *Cdkl5⁻ᐟʸ* mice.

Methylphenidate relieves hyperlocomotion in *Cdkl5⁻ᐟʸ* mice

MPH, branded as Ritalin, Concerta, Daytrana, Methylin, and Aptensio, is the most frequently used pharmacological treatment in children with ADHD. MPH functions to inhibit the reuptake of dopamine through DAT, hence increases synaptic levels of

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dopamine (41, 43). As a psychostimulant, MPH's paradoxical calming effect in treating ADHD symptoms remains puzzling. Given that loss of CDKL5 increased level of DAT in the ST-r, we reasoned that MPH treatment may target and suppress DAT function to tranquilize $Cdkl5^{-/y}$ mice. We thus evaluated the behavioral effect of MPH by testing these mice in an open field arena immediately after subcutaneous administration of MPH, with mice injected with saline as the control.

We found that saline-treated $Cdkl5^{-/y}$ mice showed hyperlocomotion, compared to WT mice (12803.4 ± 501.3 in KO vs. 7116.5 ± 262.5 in WT, $p < 0.05$; Fig. 4A, black lines in 4B), consistent with our previous findings (17). Upon administration of MPH at a low dose of 1 mg/kg, $Cdkl5^{-/y}$ mice, but not WT controls, exhibited further elevated hyperlocomotion (18334.6 ± 1097.0 in KO vs. 7765.1 ± 333.6 in WT, $p < 0.001$; Fig. 4A, gray lines in 4B), suggesting that CDKL5 deficiency increases sensitivity to MPH. Upon administration of MPH at a high dose of 30 mg/kg (MPH-30), intriguingly, the hyperlocomotion in $Cdkl5^{-/y}$ mice was significantly alleviated (KO: 8508.7 ± 339.6 with MPH-30 vs. 12803.4 ± 501.3 with saline, $p < 0.05$; Fig. 4A, red line in 4B), while locomotion in WT controls was enhanced (WT: 13373.7 ± 1059.5 in MPH-30 vs. 7116.5 ± 262.5 in saline, $p < 0.01$; Fig. 4A, blue line in 4B). These results suggest that high dose of MPH enhances locomotion in WT mice but ameliorates hyperlocomotion in $Cdkl5^{-/y}$ mice. However, locomotion of MPH-treated WT mice was returned to the baseline level in the later period (Fig. 4B, blue line). To elucidate the prolonged effects of MPH on locomotion and neurotransmission, we next performed microdialysis study on MPH-treated mice for 1 hour.

Relief of hyperlocomotion is concomitant with further increase of synaptic dopamine in the ST-r

The extracellular levels of dopamine were measured from the ST-r of MPH-treated *Cdkl5*$^{-/y}$ and their littermate controls during the open field test. In the first 15 min, MPH enhanced locomotion in both *Cdkl5*$^{-/y}$ and WT mice (Fig. 5A) without significant increase of dopamine in the ST-r (Fig. 5B). Upon the administration of MPH-30 for 15-30 min, the dopamine content in the ST-r of WT controls reached to a plateau (Fig. 5B), and these mice exhibited constant increase in locomotor activity since after (Fig. 5A). By contrast, the levels of dopamine in the ST-r of *Cdkl5*$^{-/y}$ mice continuously increased and reached to the peak at 30-45 min after application of MPH-30 (Fig. 5B), concurrently these mice exhibited reduction of locomotion that down to the baseline level (Fig. 5A). A significant reduction of locomotion (2052.7 ± 313.9 in KO vs. 3910.5 ± 580.6 in WT, $p < 0.01$; Fig. 5A, C) and increase of dopamine were observed in *Cdkl5*$^{-/y}$ mice compared to WT mice at 30-45 min after MPH-30 treatment (% of saline : 444 ± 47% in KO vs. 270 ± 26% in WT , $p < 0.05$; Fig. 5B, D), suggesting that further increase of dopamine in *Cdkl5*$^{-/y}$ mice might trigger down-stream signaling alterations leading to the relief of hyperlocomotion during this period. We thus collected the striatal tissues at 40 min after MPH-30 administration to examine the expression and phosphorylation of dopamine signaling proteins.

Methylphenidate alters DARPP32 phosphorylation in the striatum

Previous studies demonstrated that DARPP32 is a key signaling integrator enriched in the striatum (32). Phosphorylation of DARPP32 at threonine 34 (pD32-T34) and threonine 75 (pD32-T75), regulated by dopamine-mediated activation/inactivation of protein kinase A (PKA), is pivotal for psychomotor control (44). Given that loss of

CDKL5 strongly disrupts the kinome profile of PKA in the striatum (14), we questioned if DARPP32 phosphorylation would be influenced in the striatum of $Cdkl5^{-/y}$ mice, and if these alterations can be reversed by MPH. To examine this possibility, we measured the protein expression and phosphorylation of DARPP32 in the striatum of saline or MPH-treated $Cdkl5^{-/y}$ and WT littermate mice.

We found that pD32-T75 was increased in the ST-r in $Cdkl5^{-/y}$ mice compared to WT littermate control (1.49 ± 0.18 in KO vs. 1.0 ± 0.06 in WT, $p < 0.05$; Fig. 6A, C). The increased level of pD32-T75 was significantly reduced in the presence of MPH-30 (0.68 ± 0.12 in KO vs. 1.09 ± 0.08 in WT, $p < 0.05$; Fig. 6A, C), paralleled with the suppression effects of MPH-30 on locomotion of $Cdkl5^{-/y}$ mice (Fig. 4A). Given that the levels of DARPP32 total protein was not altered (Fig. 6C'), the phosphorylated proportion of pD32-T75 in the ST-r was elevated in saline-treated $Cdkl5^{-/y}$ mice ($p < 0.05$) but reduced in MPH-treated ones ($p < 0.01$; Fig. 6C"). Notably, the changes of pD32-T75 were not found in the ST-c of $Cdkl5^{-/y}$ mice ($p > 0.05$; Fig. 6D-D"). As for the levels of pD32-T34, no significant alteration was observed in both the ST-r and ST-c in $Cdkl5^{-/y}$ mice (Fig. 6B, E-F"). However, MPH-30 treatment increased pD32-T34 levels in WT mice only in the ST-c, but not in the ST-r   (Ratio to saline: 1.29 ± 0.08 in ST-c vs. 1.05 ± 0.05 in ST-r; Fig. 6B, F). The proportion of pD32-T34 was also increased in the ST-c of MPH-treated WT mice (Ratio to saline: 1.34 ± 0.11 in ST-c vs. 1.13 ± 0.06 in ST-r; Fig. 6E", F"). These findings suggest that increase of pD32-T34 in the ST-c may contribute to MPH-induced locomotor enhancement in WT mice.

CDKL5 in dopaminergic neurons is required to maintain normal locomotion

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In the striatum, DARPP32 is predominantly expressed in the postsynaptic dopamine-receptive neurons those are classified into direct (D1R-expressing) and indirect (D2R-expressing) pathways; while DAT is a protein exclusively expressed in the presynaptic dopaminergic neurons. It is unclear whether CDKL5 plays an essential role in dopaminergic and/or dopamine-receptive neurons, responsible for the locomotor hyperactivity in $Cdkl5^{-/y}$ mice. To dissect the pre-synaptic versus post-synaptic effects of CDKL5 deficiency in the striatum, we analyzed locomotor behavior in cKO mice, in which CDKL5 was ablated selectively in DAT-expressing dopaminergic neurons (driven by DAT-Cre) or the dopamine-receptive neurons expressing D1R (driven by D1R-Cre) or D2R (driven by A2aR-Cre) (45, 46).

We found that the robust novelty-driven hyperlocomotion in male $Cdkl5^{-/y}$ mice at 4-5 weeks of age (6331.2 ± 186.1 cm in KO vs. 3554.8 ± 146.9 cm in WT, $p < 0.001$, Fig. 7A, E)(17) was recapitulated in mice carrying $Cdkl5$ deletion in DAT-expressing dopaminergic neurons (DAT-cKO: 4343.6 ± 311.9 cm vs. 2796.8 ± 146.9 cm in controls, $p < 0.001$, Fig. 7D, H). By contrast, loss of CDKL5 in dopamine-receptive neurons, either in D1R-expressing neurons or in D2R-expressing neurons, did not interfere locomotion of mice (D1R-cKO: 3587.4 ± 150.7 cm vs. 3619.8 ± 208.1 cm in controls, $p > 0.05$, Fig. 7B, F; A2aR-cKO: 3293.6 ± 210.0 cm vs. 3600.6 ± 121.7 cm in controls, $p > 0.05$, Fig. 7C, G). To explore the anxiety-related behaviors in the tested mice, we measured the ratio of time spent in the central versus peripheral arena for DAT-cKO mice and their littermate controls during the open field test. The results showed no difference between genotypes (0.30 ± 0.04 in cKO vs. 0.28 ± 0.03 in Flox), consistent to our previous observation in $Cdkl5^{-/y}$ mice (Jhang et al., 2017). Therefore, our findings

suggest that CDKL5 in dopaminergic neurons is required to maintain normal locomotion without perturbing anxiety-related behaviors.

To examine the possibility that CDKL5 may regulate dopamine release and DAT phosphorylation in a cell-autonomous manner, we performed microdialysis and immunoblotting, respectively, in dopaminergic cKO mice. The results showed that DAT-cKO mice, similar to $Cdkl5^{-/y}$ mice (Fig. 2B, B', D), contained increased extracellular level of dopamine in the first dialysate from the ST-r compared to littermate controls (477.7 ± 115.7 % of control, $p < 0.05$; Fig 8A, A', B). In addition, significant down-regulation of DAT protein levels, but not pDAT levels, was found in the ST-r of DAT-cKO mice compared to the Flox controls (DAT: 66.6 ± 2.1 % of Flox, $p < 0.001$; pDAT: 97.3 ± 5.0 % of Flox, $p > 0.05$; Fig 8C, D). Intriguingly, the reduced expression of DAT and increased pDAT/DAT ratio in cKO mice (145.9 ± 6.4 % of Flox, $p < 0.001$; Fig. 8E) are opposite to those found in $Cdkl5^{-/y}$ mice (Fig. 2), suggesting that CDKL5 deficiency induced hyperlocomotion and increase of synaptic dopamine at the ST-r is likely mediated by either the cell-autonomous effect (i.e. in DA-cKO mice) or the composite effect at the entire circuit level (i.e. in $Cdkl5$ null mice).

DISCUSSION

CDKL5 deficient mice exhibit a variety of behavioral phenotypes, mimicking several key symptoms of CDD. Among them, the hyperlocomotion and learning deficits in these mice also resemble common ADHD symptoms (17). To investigate the underlying signaling pathways responsible for hyperlocomotion shared between CDD and ADHD, we first analyzed synaptic level of dopamine in the present study. We found that loss of

CDKL5 increased extracellular dopamine level in the ST-r, where the proportion of pDAT was reduced and phosphorylated DARPP32 at T75 was increased. Upon treatment of MPH, the hyperlocomotion of CDKL5 deficient mice was alleviated, along with further increase of synaptic dopamine and reduction of pD32-T75 in the ST-r. To dissect the cellular origin of CDKL5 deficiency-induced hyperlocomotion, we carried out cKO studies, ablating CDKL5 in pre- or post-synaptic parts of dopaminergic synapses. We found that selective deletion of CDKL5 in dopaminergic, but not dopamine receptive neurons, reproduced the phenotype of hyperlocomotion observed in CDKL5 deficient mice. Our findings highlight the indispensable role of CDKL5 in normalizing synaptic dopamine tone and movement control. However, given that DAT phosphorylation was not altered by CDKL5 deficiency either in dopaminergic neurons alone (DAT-cKO) or in the whole brain (*Cdkl5*$^{-/y}$), our results suggest that CDKL5-mediated movement control is most likely dependent on the composite regulation on DAT protein expression rather than DAT phosphorylation. Consistently, loss of CDKL5 increases the protein expression of tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis, in the rostral forebrain (17). Therefore, CDKL5, localized in both cytosol and nucleus (5), may take part in movement control through regulating gene expression of dopaminergic proteins at the levels of transcription, translation or via epigenetic control by phosphorylating the regulator proteins (47).

Paradoxical calming effects of MPH

MPH has been prescribed as the primary medication for ADHD. We found that mice lacking CDKL5 show enhanced sensitivity to MPH treatment comparing with WT littermate controls, at low dose (1mg/kg; Fig. 4A). This indicates that the "threshold" for

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behavioral sensitization triggered by psychostimulants (eg. MPH) was reduced in hyperactive CDKL5 deficiency mice, supporting the notion that ADHD patients have increased tendency to develop addictive disorders (48). In addition, we found that there appear to be two phases of augmentation of extracellular dopamine at the onset and offset of hyperlocomotion. The initial increase of extracellular dopamine in the ST-r of *Cdkl5*$^{-/y}$ mice (Fig. 2D) may result from impaired dopamine reuptake caused by decreased proportion of pDAT (Fig. 3C). The increased synaptic dopamine may activate the dopamine receptors leading to increase of pD32-T75 (Fig. 6A, C)(49) and enhancement of locomotor activity (Fig. 2C, 4A). Upon MPH-30 treatment, WT mice showed increased pD32-T34 in the ST-c (Fig. 6F) and enhanced locomotor activity (Fig. 4, 5A), consistent with previous reports (50). In *Cdkl5*$^{-/y}$ mice, by contrast, the synaptic dopamine in the ST-r was further increased by MPH (Fig. 5B), accompanied by reduction of pD32-T75 (Fig. 6C) (49) and alleviation of hyperlocomotion (Fig. 4, 5A). Therefore, the restoration of deregulated phosphorylation of DARPP32 in the striatal neurons may play a key role in the paradoxical calming effects of MPH. Our finding of MPH-enhanced extracellular dopamine levels was consistent with previous reports in human studies (51, 52). Of note, MPH can also block the norepinephrine transporters, although with the binding affinity of about one-fifth compared to its binding with DAT (53, 54). The potential contribution of norepinephrine in response to MPH could be involved as well. Given that hyperlocomotion is a cardinal symptom in ADHD that can be treated by MPH, our findings that MPH alleviates hyperlocomotion in CDKL5-deficient mice allow these mice to fulfill the criteria of "predictive validity" and serve as an animal model to study ADHD or related disorders with hyperactivity as a symptom.

Role of DARPP32 phosphorylation in control of locomotor activity

Protein phosphorylation of DARPP32 has been reported on various residues, including threonine 34 and threonine 75, which are phosphorylated primarily by PKA and cyclin-dependent kinase 5 through activation of D1R and D2R, respectively (44). Phosphorylation of DARPP32 at T34 results in inhibition of protein phospotase-1 (PP1) and altered activities of other kinases and phosphatases, such as mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathway, those are important for cell proliferation, neuronal signaling and gene expression (55, 56). By contrast, phosphorylation of DARPP32 at T75 dampens phosphorylation of T34 and shifts the balance of PP-1-mediated dephosphorylation (35, 57).

Our findings that increased extracellular dopamine levels and pD32-T75 in the ST-r of *Cdkl5*[-/y] mice (Fig. 2D and 6C) suggest an enhanced D2R signaling in the ST-r of those hyperactive mice. However, previous studies reported that loss of DARPP32 in D2R-expressing neurons enhances locomotion, indicating that DARPP32 is required for D2R signaling to suppress locomotion (58). Given that the protein expression of D1R and D2R was unaltered in *Cdkl5*[-/y] mice (unpublished data), the discrepancy is likely explained by the possibility that loss of CDKL5 increased the pD32-T75 through pathways other than D2R activation. Given that DARPP32 is an integrator that orchestrates the dopamine and glutamate neurotransmission in response to external stimuli (35, 59), and CDKL5 deficiency deregulates glutamatergic neurotransmission, such as increased AMPA and NMDA receptor-mediated excitatory postsynaptic current (7, 19, 20), it is likely that aberrant glutamate signaling may play a role in modulating DARPP32 phosphorylation as well. The causal relationship between altered neurotransmission, DARPP32 phosphorylation and CDKL5-modulated locomotion remains to be further investigated.

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Role of CDKL5 in ADHD

Although mice lacking CDKL5 fulfill the "predictive validity" as an animal model of ADHD (18), genetic alterations of CDKL5, however, were rarely reported in patients with ADHD (60). The genetic linkage of CDKL5 to ADHD is neither supported by a recent genome-wide association study (61). Despite of lacking direct association, the possibility that ADHD-related pathways interact with the substrates of CDKL5 cannot be excluded. Intriguingly, a set of microtubule-associated proteins (MAPs) has recently been identified as the direct substrates of CDKL5 (21, 22). Among these newly identified substrates of CDKL5, MAP1S interacts with NMDA receptor subunit and anion transporter to determine their surface localization (62, 63). CDKL5-mediated phosphorylation of MAP1S is required to maintain microtubule stability and anterograde trafficking in developing cortical neurons (21). Our finding that CDKL5 was required in dopaminergic neurons to suppress hyperlocomotion (Fig. 7) raise a possibility that CDKL5 deficiency may affect trafficking of DAT from the cell bodies of dopaminergic neurons to the terminals at the striatum, disturb the homeostasis of dopamine neurotransmission and eventually impair the convergent pathways for motor control shared by ADHD and CDD.

CDD vs. RTT

CDD were initially identified as a variant of Rett syndrome (RTT, OMIM 312750; ICD-10-CM code: F84.2), partly due to similar symptoms, including stereotypic hand movement (64, 65). With distinct genetic etiology, more than 95% of RTT patients are caused by mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2, OMIM 300005)(66). Therefore, CDD has been classified as an independent

clinical entity recently (ICD-10-CM code: G40.42) (67, 68). In view of psychomotor function in mouse models for RTT and CDD, *Mecp2*$^{-/y}$ mice exhibit significant motor deficit of hypolocomotion (69-71), intriguingly opposite to hyperlocomotion in *Cdkl5*$^{-/y}$ mice (17) (and this study). In parallel with the behavioral phenotypes, the dopamine content in the ST-r is reduced in *Mecp2*$^{-/y}$ mice, but increased in *Cdkl5*$^{-/y}$ mice (17, 70). The expression of DAT is elevated in the ST-r of *Cdkl5*$^{-/y}$ mice (Fig. 3), but is not altered in caudate nucleus of RTT patients (72). Moreover, selective deletion of MeCP2 or CDKL5 in GABAergic or dopaminergic neurons, respectively, recapitulates the locomotor activities observed in the correspondent null mice (26, 71)(Fig. 7). These findings suggest that deficiency of MECP2 or CDKL5 may generate symptoms in RTT or CDD through independent mechanisms, at least in the aspect of psychomotor control. Clinically, numerous CDD patients cannot walk independently (73), which is, however, not recapitulated in current mouse models of CDD (14-16). The discrepancy is likely due to fundamental differences between species. Establishing non-human primate model for CDD may reveal the evolutionary functional divergence of CDKL5 in the brains of different species.

In summary, the present study showed that MPH treatment alleviated the hyperlocomotion in CDKL5 deficient mice, and the behavioral amelioration by MPH was associated with restoration of protein phosphorylation of DARPP32 in the ST-r. We also dissected the cellular origin of CDKL5-mediated regulation of locomotor activity, revealing that CDKL5 functions cell-autonomously in dopaminergic neurons, but not in dopamine receptive neurons. Our findings suggest that CDKL5 is essential to control locomotor behavior by regulating region-specific dopamine content and phosphorylation of dopamine signaling proteins in the striatum. The direct, as well as indirect, target

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proteins regulated by CDKL5 may play a key role in movement control and the

therapeutic development for hyperactivity disorders.

MATERIALS AND METHODS

Animals

Male *Cdkl5* null (*Cdkl5$^{-/y}$*) mice were generated by crossing C57BL/6 J male mice

(National Laboratory Animal Center, Taiwan) to heterozygous *Cdkl5* females [*Cdkl5$^{+/-}$*,

B6.129(FVB) *Cdkl5$^{tm1.1Joez}$*/J, the Jackson Laboratory], whose kinase domain of CDKL5

was truncated by a premature STOP codon resulting in an exon 6 deletion and loss of

function of CDKL5 protein (14). The cKO mice were generated by crossing

heterozygous females of floxed *Cdkl5* mice (B6.129-*Cdkl5$^{tm1.2Joez}$*/J, the Jackson

Laboratory) with male mice of different Cre lines, including DAT-Cre

[B6.SJL-*Slc6a3$^{tm1.1(cre)Bkmn}$*/J, the Jackson Laboratory], D1R-Cre [Tg(Drd1a-cre)

EY217Gsat/Mmucd, MMRRC] and A2aR-Cre [Tg(Adora2a-cre)KG139Gsat/Mmucd,

MMRRC]. All mice were bred and housed in the individually ventilated cages (IVC,

Alternative Design, USA) at 22 ± 2 °C and $65 \pm 15\%$ humidity under a 12-h light-dark

cycle (light on 08:00 to 20:00). Irradiated diet (#5058, LabDiet, USA) and sterile water

were supplied *ad libitum*. All mice were weaned and ear-tagged at P21-23, and then

genotyped as previously described for *Cdkl5* null mice (17). The protocols listed on the

following websites were used for genotyping of cKO mice: floxed Cdkl5

(https://www.jax.org/strain/030523); DAT-Cre (https://www.jax.org/ strain/006660);

D1R-Cre (https://www.mmrrc.org/catalog/sds.php?mmrrc_ id=30778) and A2aR-Cre

(https://www.mmrrc.org/catalog/sds.php?mmrrc_id=31168). The littermate mice

containing either *flox* or *Cre* allele (Flox: *Cdkl5$^{flox/y; +/+}$* or Cre: *Cdkl5$^{+/y; Cre/+}$*), or none

of them (WT: *Cdkl5$^{+/y; +/+}$*), served as the controls of cKO mice (*Cdkl5$^{flox/y; Cre/+}$*).

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Experiments described in this study were approved by the Institutional Animal Care and
Use Committee at National Cheng-Chi University.

In vivo microdialysis

Male *Cdkl5*$^{-/y}$ mice and their littermate wild-type controls at 6-8 weeks of age were
anesthetized with isoflurane (100 ml/min; VIP 3000, Midmark, USA) and implanted
with a unilateral guiding cannula to the right ST-r (AP +1.6 mm; ML -1.5 mm; DV -2.7
mm) (74) by using stereotaxic surgery (Model 963, David Kopf Instruments, USA).
After 36 hours of recovery, the mice were connected to a concentric probe (AZ-X-Y,
EiCom, Japan) and a free-moving swivel system (TSU-20, EiCom) to perform
microdialysis as described (75). Ringer's solution (147 mM NaCl, 4 mM KCl, 1.5 mM
CaCl$_2$, pH 7.4) was perfused into the dialysis probe with a micropump (KDS-101, Kd
Scitific, USA) at a constant flow rate of 1 μl/min. The dialysates were collected every 15
min for a real-time analysis of dopamine level (See below, Fig. 1).

Quantification of dopamine (DA)

Perfusates of 10 μl were manually injected into the high-performance liquid
chromatography (HPLC) system (HTEC-500, EiCom, Japan) through a Hamilton
syringe immediately after collection every 15 min. DA in the dialysate was separated
through a reverse-phase ODS column (PP-ODS, EiCom) with the mobile phase of
sodium phosphate buffer (0.1M) containing sodium decanesulfonate (DSS; 500 mg/L,
Naclai, Japan), sodium ethylene-diamine-tetra-acetate (EDTA-2Na; 50 mg/L, Dojundo,
Japan) and 1% methanol (Merck). The HPLC apparatus and the working electrode
(WE-3G, EiCom, Japan) were stabilized by pre-running the mobile phase for >180 min
at the flow rate of 500 □l/min prior to measuring the DA standards and then samples.

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The levels of extracellular DA contained in the dialysates were determined by calculating the integral area of the DA peak (PowerChrom, USA) that appears at the retention time similar to that of DA standards. To set up the standard curve, pure compounds of dopamine hydrochloride (Sigma) were dissolved in hydrochloric acid (0.1M) and diluted to final concentrations with 0.1M phosphate buffer. The released DA levels in *Cdkl5* null mice or in DAT-cKO mice were shown as "DA peak area" and compared to WT mice (Fig. 2 and 8). The effects of MPH on DA release during the 60 min infusion period after drug administration were shown as "% of baseline" levels (Fig. 5).

Open field test

For methylphenidate (MPH) administration, one tablet of methylphenidate hydrochloride (10 mg, Novartis) was dissolved in 1 ml sterile saline and the supernatant was collected following centrifugation at 13,000 rpm for 5min (RA 2024, Kubota, Japan). Mice at 4–5 weeks of age were subjected to subcutaneous injection of saline or MPH at the dose of 1 or 30 mg/kg (~0.2 ml) immediately prior to an open field test as previously described (70). For open field test during microdialysis, all tested animals were habituated in a clear Plexiglas container (40 cm in diameter, 32 cm in height, the "bowl") for 40 min on the day before testing. On the testing day, these mice were subjected to a "naïve" trial for 18 min in the bowl before hooking up the probes, and then tested with microdialysis for 2.5 hours after the start of pumping. An independent cohort of mice was tested in an open field square (40 x 40 x 25 cm) for 34 min to test the effect of MPH. The same square arena was also used to examine locomotion of cKO mice, as *Cdkl5* null mice tested previously (17). The testing field was videotaped with an overhead camera upon dim light. Total distance traveled of locomotor activity was

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analyzed every 15 min for microdialysis with the Smart® video-tracking system (Harvard Apparatus, USA). The testing results by square arena were analyzed for 30 min (from 3.5 to 33.5 min, excluding the first 3.5 min as the habituation period) or 12 min (from 3.5 to 15.5 min). Body movements slower than 2 cm/s were counted as resting. The anxiety-related behaviors were analyzed by measuring the ratio of time spent in the central versus peripheral arena. The comparison between genotypes and treatments was conducted by using Student's *t*-test or two-way ANOVA followed by Bonferroni's *post-hoc* test (Prism 8, GraphPad Inc.). All the behavioral assays were performed in a soundproofed room at the same period of a day (1:00 to 6:00 pm) by operator blind to genotype of tested mice.

Western blotting

Brain tissues were harvested from the ST-r and ST-c of *Cdkl5⁻/ʸ* and WT littermate mice at the age of 4-5 weeks. The tissues were homogenized by sonication in lysis buffer containing 1% protease inhibitors and phosphatase inhibitor (Sigma), and then the proteins were extracted for immunoblotting as described (71). Briefly, twenty micrograms of protein were separated by polyacrylamide gel electrophoresis (10%, Bio-Rad) with 70 V for 30 min followed by 110 V for 2h. Proteins were transferred to a PVDF membrane (Millipore) by liquid electroblotting (Mini Trans-Blot Cell, Bio-Rad) with 350 mA for 2 h. The membranes were blocked by skim milk (5% in Tris-buffered saline containing 0.1% Tween 20) and incubated with primary antibodies against dopamine transporter (DAT, 1: 2000, Millipore), phospho-DAT at threonine 53 (pDAT, 1: 2000, Abcam), DARPP32 (1:50,000, Cell Signaling), phospho-DARPP32 at threonine 34 (pD32-T34, 1:5000, Sigma), phospho-DARPP32 at threonine 75 (pD32-T75, 1:5000, Cell Signaling) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:100,000,

Millipore) at 4 °C for 16 h. Following incubation with peroxidase-conjugated secondary antibodies for 2 h, these blots were detected using an image acquisition system (Celvin®S420, Biostep, Germany) upon incubation with an enhanced chemoluminescence reagent (ECL, Millipore). The intensity of targeted protein expression was normalized with that of GAPDH by densitometry-based quantification (Image J, NIH). The expression levels of targeted proteins were presented as "ratio to WT". The differences between genotypes and treatments were compared by using two-way ANOVA followed by Bonferroni's *post-hoc* test (Prism 8, GraphPad Inc.).

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. Diagram illustrates the experimental design for *in vivo* microdialysis.

"Pump on" indicates the starting time for infusion of Ringer's solution into the rostral

striatum. Because of the dead volume (~10 μl) in the tethering system, the initial

dopamine (DA) content in the first dialysate (-15 ~ 0 min) reflects the synaptic level of

dopamine during open field test (OFT) immediately after infusion. Each animal was

next treated with saline subcutaneously and followed by OFT measurement of

locomotion for 1 h, during which the dialysate was collected every 15 min started from

10 min after saline injection. Methylphenidate (MPH, 30 mg/kg) administration was

followed immediately, and the procedures for measuring DA and locomotion were

performed similarly as conducted during the saline–treated period. The numbers and

alphabets shown in parentheses indicate the corresponding figures of the results.
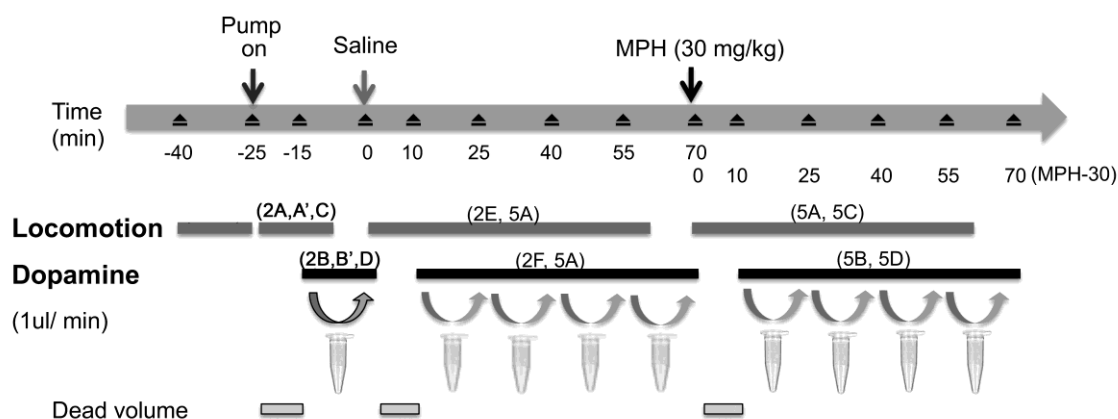

Figure 1 (Liao)

Figure 2. Hyperlocomotion and increased extracellular dopamine in the ST-r of
Cdkl5$^{-/y}$ **mice.** Male mice at 6-8 weeks of age were implanted with cannula for
microdialysis probes at the ST-r and subjected to the measurement of dopamine level
while performing open field test. *Cdkl5$^{-/y}$* mice travel longer distance with concurrently
increased levels of extracellular dopamine (DA, peaks in gray) in the ST-r (B, B', D)
compared to wild-type (WT) mice (A, A', C). Continuous infusion of Ringer's solution
reduces local DA levels, which in turns diminishes the differences of locomotor activity
between genotypes. * $p < 0.05$, ** $p < 0.01$, Student-*t* test.

Figure 2 (Liao)
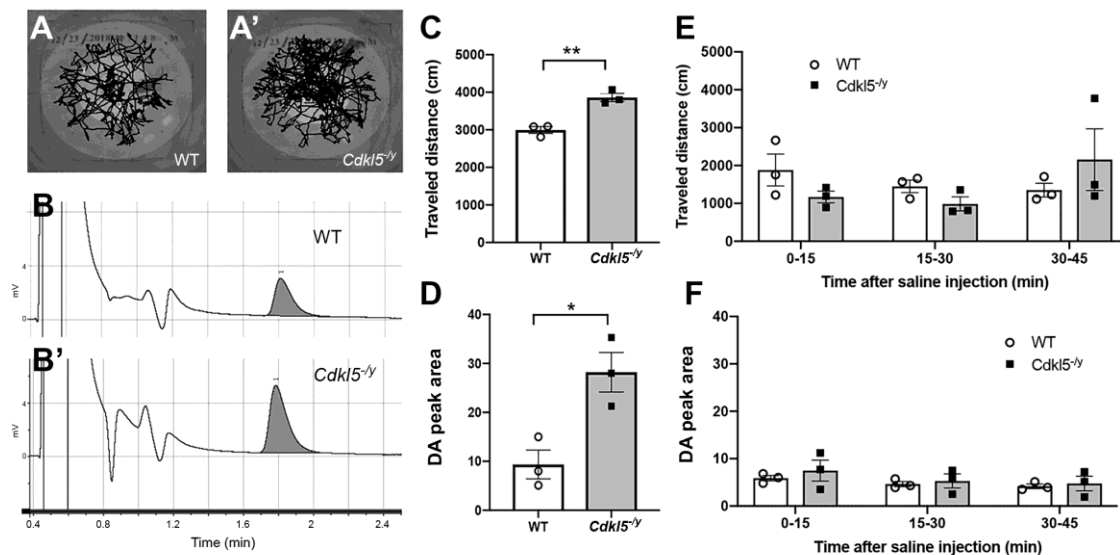
Figure 3. Altered expression and phosphorylation proportion of dopamine transporter in *Cdkl5*^{-/y} mice. Brain tissues of the striatum from the rostral (ST-r) and caudal (ST-c) parts in male mice at 4-5 weeks of age were harvested and protein levels of DAT and phosphorylated DAT at threonine 53 (pDAT) were quantified by immunoblotting (A, A'). Increased DAT and reduced proportion of pDAT were observed in the ST-r, while opposite alterations occurred in the ST-c (B, C). $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$, two-way ANOVA followed by Bonferroni's *post-hoc* test.

Figure 3 (Liao)
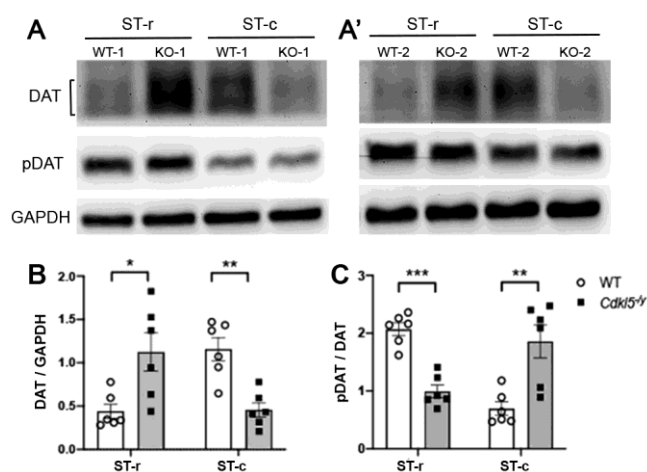
Figure 4. Effects of methylphenidate on locomotor activity. Male wild-type (WT) and *Cdkl5$^{-/y}$* (KO) mice at 4-5 weeks of age were assessed with open field test for 30 min after subcutaneous administration of MPH or saline. A and B show the dose-dependent and time-dependent effects of MPH, respectively. * $^{(\#)}$ $p < 0.05$, ** $^{(\#\#)}$ $p < 0.01$, *** $^{(\#\#\#)}$ $p < 0.001$; *, compared to WT control treated with the same dose of MPH; $^{\#}$, compared to saline-treated mice of the same genotype; two-way ANOVA followed by Bonferroni's *post-hoc* test.
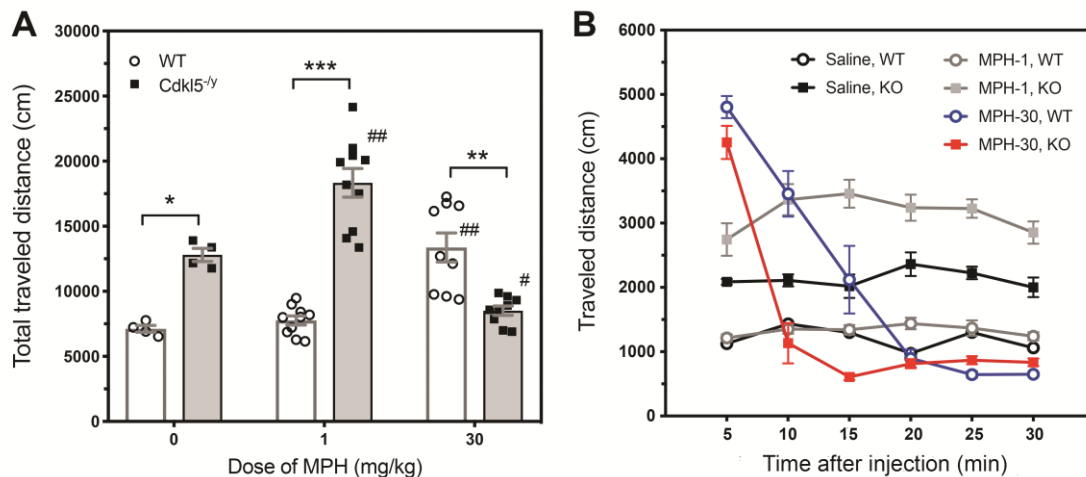

Figure 4 (Liao)

Figure 5. Alleviated hyperlocomotion is concomitant with elevated level of dopamine in the ST-r of MPH-treated *Cdkl5$^{-/y}$* mice. Cerebrospinal fluid was collected from the rostral striatum through a microdialysis probe every 15 min after subcutaneous injection of saline (Sal), followed by MPH (30mg/kg, MPH-30) in free-moving mice with or without CDKL5 expression (refer to Fig. 1). The locomotion of these mice was measured by an open field test (A) and the extracellular dopamine level in dialysates was determined by HPLC in parallel (B). At 30-45 min after MPH administration, a reduction of locomotion and an increase of dopamine were found in *Cdkl5$^{-/y}$* mice compared WT mice (C, D). * $p < 0.05$, compared to WT control, two-way ANOVA followed by Bonferroni's *post-hoc* test.
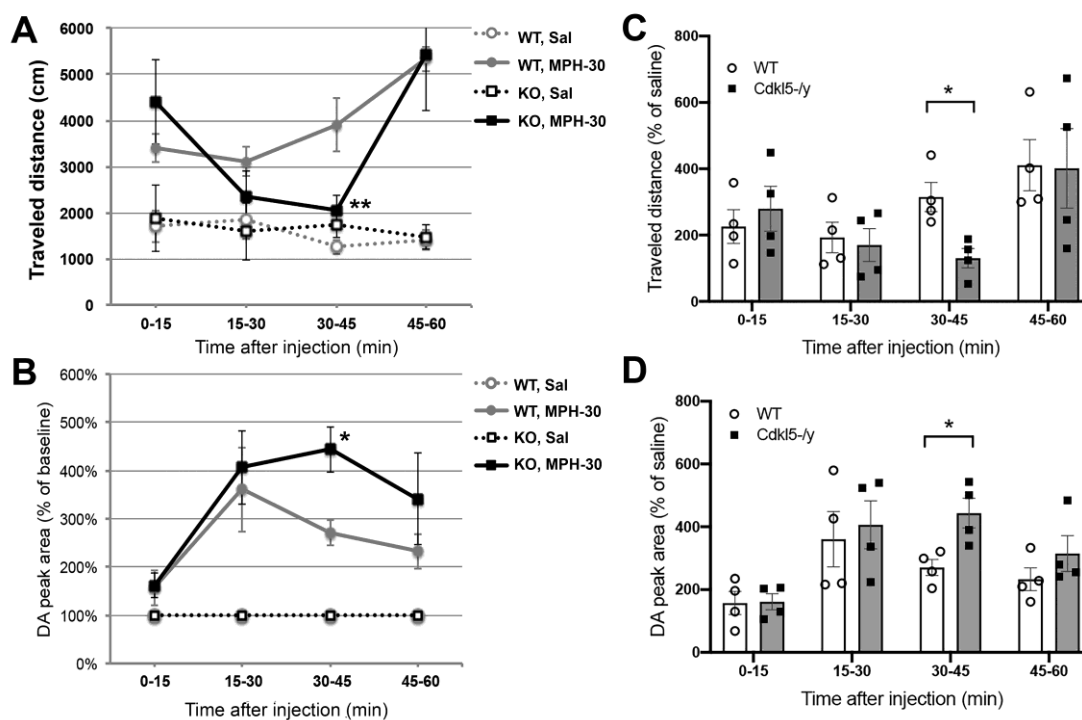

Figure 5 (Liao)

Figure 6. Effects of MPH on the protein expression and phosphorylation of DARPP32 in the striatum. The rostral (ST-r) and caudal (ST-c) striatal tissues were harvested from male *Cdkl5*$^{-/y}$ or wild-type (WT) mice at the age of 4-5 weeks 40 min after subcutaneous administration of MPH or saline. A, B: Immunoblotting results show that MPH affects the protein expression of total DARPP32 (D32) and phospho-DARPP32 at threonine 75 (pD32-T75) or at threonine 34 (pD32-T34) in the ST-r and ST-c differently with genotypes. The expression of pD32-T75 (A, C) and phosphorylated proportion of DARPP32 (C") in the ST-r is increased in saline-treated, but reduced in MPH-treated, *Cdkl5*$^{-/y}$ mice compared to WT controls. In contrast, the expression of pD32-T34 (B, F) and its phosphorylated proportion (F") in the ST-c are increased in WT mice with MPH treatment. MPH does not affect pD32-T75 and pD32-T34 at the ST-c (D-D") and ST-r (E-E"), respectively, in mice of both genotypes. The expression of total DARPP32 at both the ST-r and ST-c is not altered upon MPH treatment (C'-F'). Data show mean ± SEM. n = 3-5; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-way ANOVA with Bonferroni's *post-hoc* test.
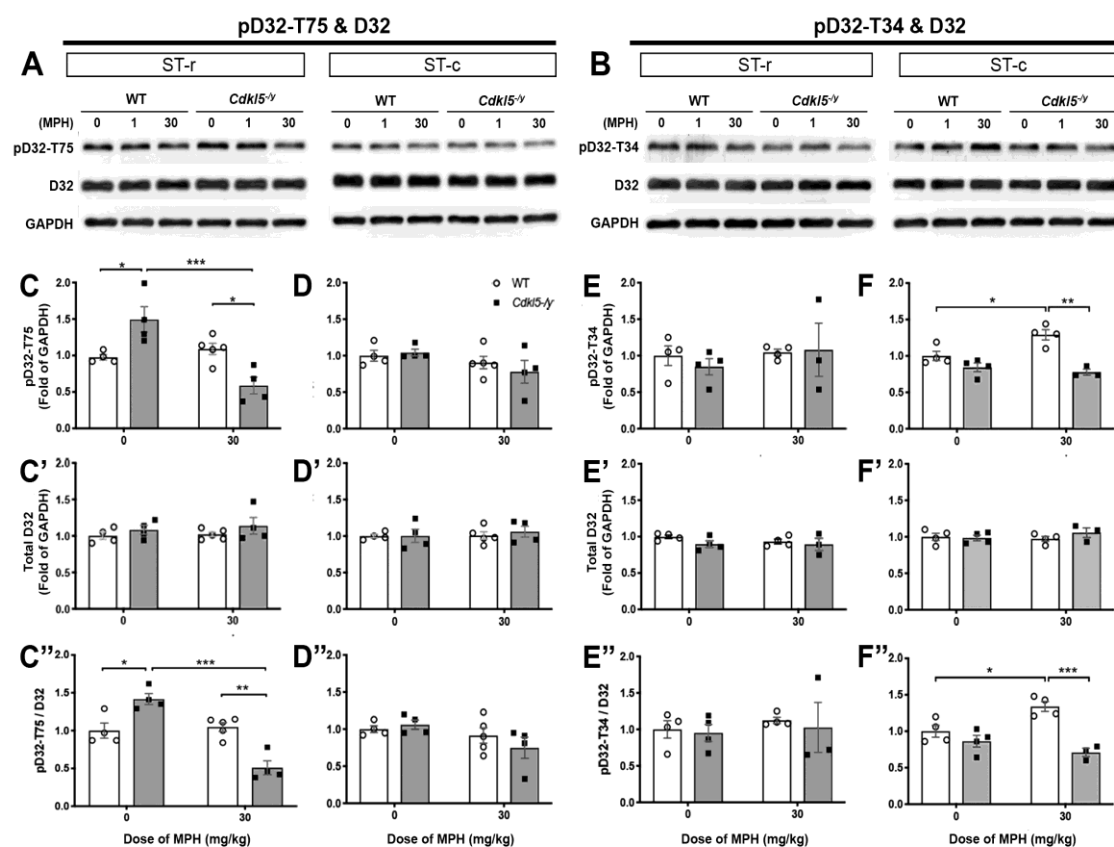
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Figure 6 (Liao)

Figure 7. Loss of CDKL5 in DAT-expressing dopaminergic neurons recapitulates hyperlocomotion in *Cdkl5*$^{-/y}$ mice. Male mice at 4-5 weeks of age were tested in an open field arena and total traveled distance was measured. The representative moving trajectory delineated in A-D were selected from the pair of tested mice whose performance was the closest to the group average. The performance of *Cdkl5*$^{-/y}$ and cKO mice was normalized with the average of their littermate wild-type (WT) mice or controls (including WT, Flox and Cre mice), respectively, and present as "ratio to WT" or "ratio to control". The tested mice were derived from six to eight litters for each line. Data show mean ± SEM. *** $p < 0.001$; Student-*t* test.
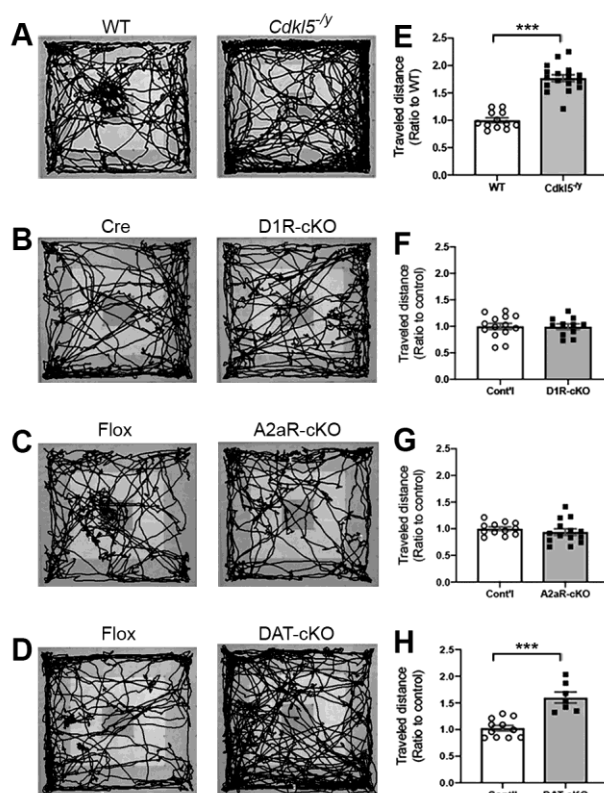

Figure 7 (Liao)

Figure 8. Loss of CDKL5 in dopaminergic neurons down-regulates DAT expression and increases dopamine release in the rostral striatum. Male DAT-cKO mice and their littermate controls at 6-12 weeks of age were subjected to dopamine measurement by microdialysis. DAT-cKO mice, similar to $Cdkl5^{-/y}$ mice (Fig. 2B, B', D), contain increased extracellular levels of dopamine at the rostral striatum (ST-r) compared to controls (A-B). Immunoblotting results show that the DAT protein expression is significantly down-regulated, while there is no change for phosphorylated DAT at threonine 53 (pDAT), in the ST-r of DAT-cKO mice compared to the Flox controls (C-E). Three pairs of tissues with indicated genotypes in C were derived from 3 different litters of mice at 4-5 weeks of age. Data show mean ± SEM. * $p < 0.05$, *** $p < 0.001$; two-way ANOVA with Bonferroni's *post-hoc* test in B and D, Student-*t* test in E.

Figure 8 (Liao)
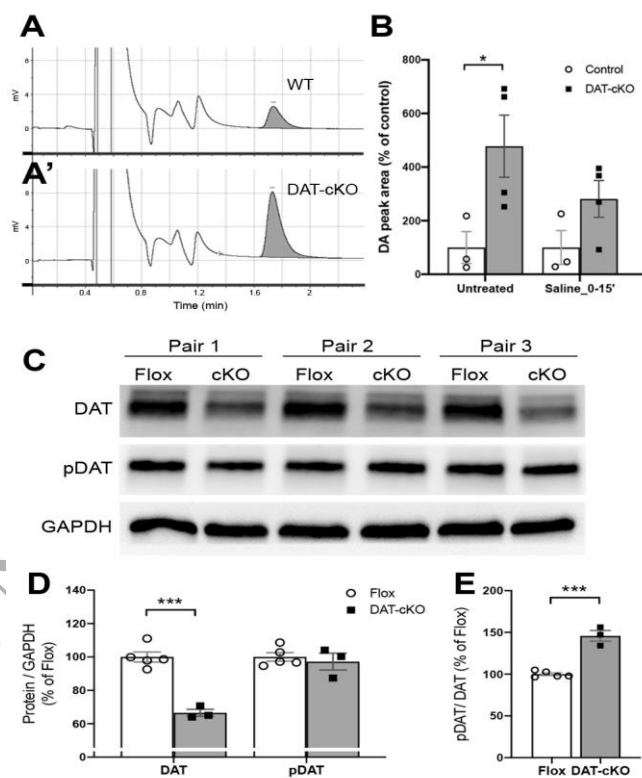
ABBREVIATION

ADHD   attention deficit hyperactivity disorder

ANOVA   analysis of variance

CDD   CDKL5 deficiency disorder

CDKL5   cyclin-dependent kinase-like 5

CTX-r   rostral cortex

D1R   dopamine D1 receptor

D2R   dopamine D2 receptor

DARPP32   dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa

DAT   dopamine transporter

GAPDH   glyceraldehyde 3-phosphate dehydrogenase

HPLC   high performance liquid chromatography

MAP1S   microtubule-associated protein 1S

MeCP2   methyl-CpG binding protein 2

MPH   methylphenidate

OFT   open field test

PBS   phosphate-buffered saline

RTT   Rett syndrome

ST-c   caudal striatum

ST-r   rostral striatum

WT   wild-type