

Aberrant regulation of synchronous network activity by the attention-deficit/hyperactivity disorder-associated human dopamine D4 receptor variant D4.7 in the prefrontal cortex

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Key points

- The hD4.7 variant has been linked to attention-deficit/hyperactivity disorder (ADHD); however, the underlying mechanism is unknown.
- We found that activation of hD4.7 induced over-suppression of glutamatergic excitatory network bursts and under-suppression of GABAergic inhibitory network bursts in the prefrontal cortex (PFC) circuitry.
- Methylphenidate, a psychostimulant drug used to treat ADHD, normalized the effects of hD4.7 on synchronous network bursts in PFC pyramidal neurons.
- The findings of the present study suggest that the aberrant regulation of PFC synchronous network activity by hD4.7 may underlie its involvement in ADHD.

Abstract A unique feature of the human D₄ receptor (hD₄R) gene is the existence of a large number of polymorphisms in exon 3 coding for the third intracellular loop, which consists of a variable number of tandem repeats. The hD₄R variants with long repeats have been linked to attention-deficit/hyperactivity disorder (ADHD); however, the underlying mechanism is unknown. Emerging evidence suggests that selective attention is controlled by the rhythmic synchronization in the prefrontal cortex (PFC) and its connected networks. In the present study, we examined the role of hD₄R variants in regulating PFC synchronous network activity. D₄R knockout mice with viral infection of hD4.4 or hD4.7 in the medial PFC were used. Whole-cell patch-clamp recordings were performed to examine the effects of activating hD4.x on the spontaneous large scale correlated activity in PFC pyramidal neurons. We found that, compared to the normal four-repeat variant hD4.4, the ADHD-linked variant hD4.7 induces more suppression of glutamatergic excitatory network bursts and less suppression of GABAergic inhibitory network bursts in the PFC circuitry. Methylphenidate, a psychostimulant drug used to treat ADHD, normalized the effects of hD4.7 on synchronous network bursts in PFC pyramidal neurons. These results reveal the differential effects of hD₄R variants on the integrated excitability of PFC circuits. It is suggested that the aberrant regulation of PFC network activity by hD4.7 may underlie its involvement in ADHD. The methylphenidate-induced normalization of synaptic circuitry regulation may contribute to its effectiveness in ADHD treatment.

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Abbreviations aCSF, artificial cerebrospinal fluid; ADHD, attention-deficit/hyperactivity disorder; AMPAR, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; APV, DL-2-amino-5-phosphonovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GABA_AR, GABA_A receptor; GFP, green fluorescent protein; hD₄R, human dopamine D₄ receptor; NMDAR, NMDA receptor; PFC, prefrontal cortex; PSD, postsynaptic density.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent and debilitating disorder diagnosed on the basis of overactivity, inattention and impulsivity. A meta-analysis suggests that ADHD is associated with significant weaknesses in several key executive functions, including response inhibition, vigilance, working memory and planning (Willcutt *et al.* 2005), all of which are controlled by the prefrontal cortex (PFC). Although the pathophysiological mechanisms underlying ADHD remain to be clarified, genetic studies have linked the dopamine D₄ receptor, which is highly enriched in the PFC (Mrzljak *et al.* 1996; Wedzony *et al.* 2000), to ADHD (Bobb *et al.* 2005; Grady *et al.* 2003; Swanson *et al.* 2007).

The gene encoding human D₄ receptor (hD₄R) contains a large number of polymorphisms in the coding region for the third intracellular loop, which consists of a variable number (2–11) of 48 bp tandem repeats (Van Tol *et al.* 1992). The three most common variants contain two, four and seven repeats (D4.2, D4.4, D4.7), with a global frequency of 8%, 64% and 21%, respectively (Chang *et al.* 1996). These proline-rich repeats provide the binding sites for other proteins containing the SH3 domain. So far, the molecular mechanism and functional significance of the remarkable variable number tandem repeat polymorphism of hD₄R is poorly understood. Importantly, several studies have reported a significant association of ADHD with the D4R gene seven-repeat allele (LaHoste *et al.* 1996; Rowe *et al.* 1998; Smalley *et al.* 1998; Faraone *et al.* 1999; El-Faddagh *et al.* 2004; Li *et al.* 2006). However, the underlying mechanism is unknown.

Emerging evidence suggests that the control of attention arises from interactions between widespread cortical and subcortical networks regulated via their rhythmic synchronization (Buschman & Miller, 2007; Gregoriou *et al.* 2009; Womelsdorf *et al.* 2010; Miller and Buschman, 2013). The synchronized network activity is an important intrinsic feature of cortical circuits (Opitz *et al.* 2002; Buzsáki & Draguhn, 2004) and plays a significant role in controlling brain functions (Varela *et al.* 2001; von der Malsburg *et al.* 2010). Key factors involved in synchronous network activity include the strength of glutamatergic input from the recurrent excitatory connections between

pyramidal neurons (Stoop *et al.* 2003; Panuccio *et al.* 2009) and the strength of GABAergic input from interneurons (Korn *et al.* 1987; Traub *et al.* 1989). Excitatory and inhibitory synaptic conductances may be exquisitely balanced in normal neuronal activity (Borg-Graham *et al.* 1998; Haider *et al.* 2006), which is critical for complex behaviours (Klausberger & Somogyi 2008; Womelsdorf *et al.* 2010; Yizhar *et al.* 2011).

An understanding of how the synchronized cortical network activity is regulated in normal and ADHD conditions is lacking. In the present study, we examined the role of hD₄R variants in regulating the integrated excitability of cortical circuits by measuring their impact on spontaneous large scale correlated activity in PFC pyramidal neurons. We show that the synchronized network activity in the PFC is more prominently suppressed by hD4.7. These results provide a potential pathophysiological basis for the frontal hypoactivity found in the diagnosis of ADHD (Dickstein *et al.* 2006; Fernández *et al.* 2009).

Methods

The generation of hD4.x viruses

The HA-tagged human D4.4 and D4.7 plasmids (Rondou *et al.* 2008) were kindly provided by Dr Kathleen Van Craenenbroeck at the Laboratory of Eukaryotic Gene Expression and Signal Transduction (LEGEST), BELGIUM. The generation of Sindbis virus used the same procedure as that described previously (Liu *et al.* 2011). Briefly, the cDNAs encoding green fluorescent protein (GFP) or GFP-hD4.x were subcloned to pSinRep5 vector (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Recombinant GFP-pSinRep5 or GFP-hD4.x-pSinRep5 was linearized with *NotI*. The DH26S plasmid was linearized using *XhoI*. The linearized templates were transcribed *in vitro* using the mMessage Machine SP6 kit (Ambion, Austin, TX, USA) and the RNAs were electroporated into baby hamster kidney cells. The extracellular medium containing the recombinant viruses was harvested after 24–48 h. The medium was concentrated on a discontinuous sucrose gradient (55% and 20% sucrose) using ultracentrifugation (160,000 g for 90 min at 4°C).

In vivo delivery of hD4.x viruses

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the State University of New York at Buffalo. D₄R knockout mice (Rubinstein *et al.*, 1997, 2001) were originally provided by Dr David Grandy at Oregon Health & Science University and later bred and maintained in our own laboratory for the experiments. *In vivo* virus-based gene delivery into medial PFC was performed as described previously (Duffney *et al.* 2015). In brief, D₄R knockout mice (2–3 months old) were anaesthetized by an i.p. injection of pentobarbital (50 mg kg⁻¹) and placed on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The Sindbis viral suspension (0.5 μ l) was injected with a Hamilton syringe (needle gauge 31) at a speed of \sim 0.2 μ l min⁻¹, and the needle was kept in place for an additional 5 min. The virus was delivered bilaterally to the medial prefrontal area of mice using the co-ordinates: 2.0 mm anterior to bregma and 0.5 mm lateral. The needle was extended to a depth of 1.3 mm below the tissue surface, and the virus was injected to each side. Animals were allowed to recover for 24–48 h after viral injection, and analgesia was provided postoperatively during the recovery.

Slice preparation

Mice (2–3 months old) were deeply anaesthetized using isoflurane and killed by decapitation. The brain was removed quickly and placed into the ice-cold sucrose solution containing (in mM): 234 sucrose, 4 MgSO₄, 2.5 KCl, 1 NaH₂PO₄, 0.1 CaCl₂, 15 HEPES and 11 glucose (pH 7.4, 300 mOsm). Coronal slices (350 μ m) were cut with a vibratome (VP1000S; Leica Microsystems, Wetzlar, Germany). Slices were incubated at 32–34°C for 1 h in artificial cerebrospinal fluid (aCSF) (in mM: 130 NaCl, 26 NaHCO₃, 1 CaCl₂, 2 MgCl₂, 3 KCl, 10 glucose and 1.25 NaH₂PO₄) bubbled with 95% O₂ and 5% CO₂. Then slices were kept at room temperature (22–24°C) in a modified aCSF solution (in mM: 2 CaCl₂, 0.1 MgCl₂ and 3.5 KCl) (Aradi & Maccaferri, 2004; Juuri *et al.* 2010; Ziburkus *et al.* 2013) for 1–2 h before recordings.

Electrophysiological recordings

The whole-cell voltage-clamp technique (Zhong & Yan, 2004, 2014; Yuen *et al.* 2012) was used to record the neuronal network currents. Slices were placed in a recording chamber and superfused with the modified aCSF solution containing low (0.1 mM) MgCl₂, high (2 mM) CaCl₂ and slightly higher (3.5 mM) KCl to keep the slices in an active state. Neurons infected with GFP-conjugated hD4.4 or hD4.7 Sindbis virus in layer V of the medial PFC were visualized with a fluorescence

microscope and selected for recordings. A Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA) and a Digidata1322A (Molecular Devices) were used. Patch electrodes were filled with the internal solution containing (in mM): 130 Cs-methanesulphonate, 4 NaCl, 10 HEPES, 0.5 EGTA, 2 QX-314, 12 phosphocreatine, 4 ATP and 0.5 GTP (pH 7.2–7.3, 265–270 mOsm). Tight seals (2–10 G Ω) were obtained by applying negative pressure. The membrane was disrupted with additional suction and the whole-cell configuration was obtained. Neurons were voltage clamped at -70 mV or -20 mV throughout the recordings. Series resistances were \sim 12 M Ω and were not compensated.

Statistical analysis

Data acquisition was carried out using Clampex, version 9 (Molecular Devices). The sampling rate was 5 kHz and the low-pass filtering frequency was 1 kHz. Each neuron was continuously recorded for 10 min, and the averaged amplitude and frequency of network bursts within a 2 min timeframe at the stable state before and after drug application were calculated. Data analyses were performed with Clampfit (Molecular Devices), Mini-Analysis (Synaptosoft Inc., Fort Lee, NJ, USA) and KaleidaGraph (Synergy Software, Reading, PA, USA) software. All data are expressed as the mean \pm SEM. Mann–Whitney *U* tests were used to determine the significance of the effects of various agents on network bursts. Experiments with two animal groups were analysed statistically using unpaired Student's *t* tests. Experiments with more than two animal groups were subjected to one-way ANOVA, followed by *post hoc* Bonferroni tests.

Results

The synchronous network activity in the PFC

Neurons in the PFC are not highly ordered like those in hippocampus, and the loss of the integrity of inhibitory and excitatory synaptic transmissions in *in vitro* preparations has often led to difficulty in uncovering synchronous network activity in PFC slices. To elevate the neuronal activity in slices to the *in vivo* level, we performed whole-cell patch clamp recordings in the modified aCSF with a lower Mg²⁺ concentration (0.1 mM) and a higher Ca²⁺ concentration (2 mM), as established previously (Aradi & Maccaferri, 2004; Juuri *et al.* 2010; Ziburkus *et al.* 2013).

In cortical slices (350 μ m), the slowly rhythmic synchronous bursting of larger numbers of neurons in the network gave rise to the spontaneous large scale correlated activity. As shown in Fig. 1A, spontaneous network bursts occurred on a background of asynchronous unitary postsynaptic currents in layer V PFC pyramidal neurons (held

at -70 mV). The network bursting-associated inward current had a mean \pm SEM amplitude of 1810 ± 172 pA ($n = 8$) and a mean \pm SEM interval of 18.3 ± 2.0 s ($n = 8$).

To examine the synaptic origin of synchronous network activity, we applied α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), NMDA receptor (NMDAR) and GABA_A receptor (GABA_AR) antagonists, respectively. As shown in Fig. 1A, the large-scale network current was blocked

by AMPAR antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) ($50 \mu\text{M}$) ($n = 5$) or NMDAR antagonist DL-2-amino-5-phosphonovaleric acid (APV) ($50 \mu\text{M}$) ($n = 6$). Inhibition of GABA_A receptors with bicuculline ($20 \mu\text{M}$) did not result in the blockade of synchronous network activity but led to the modulation of burst amplitude ($14.2 \pm 5.3\%$ increase, $n = 5$, $P < 0.05$) and frequency ($43 \pm 4\%$ decrease, $n = 5$, $P < 0.01$).

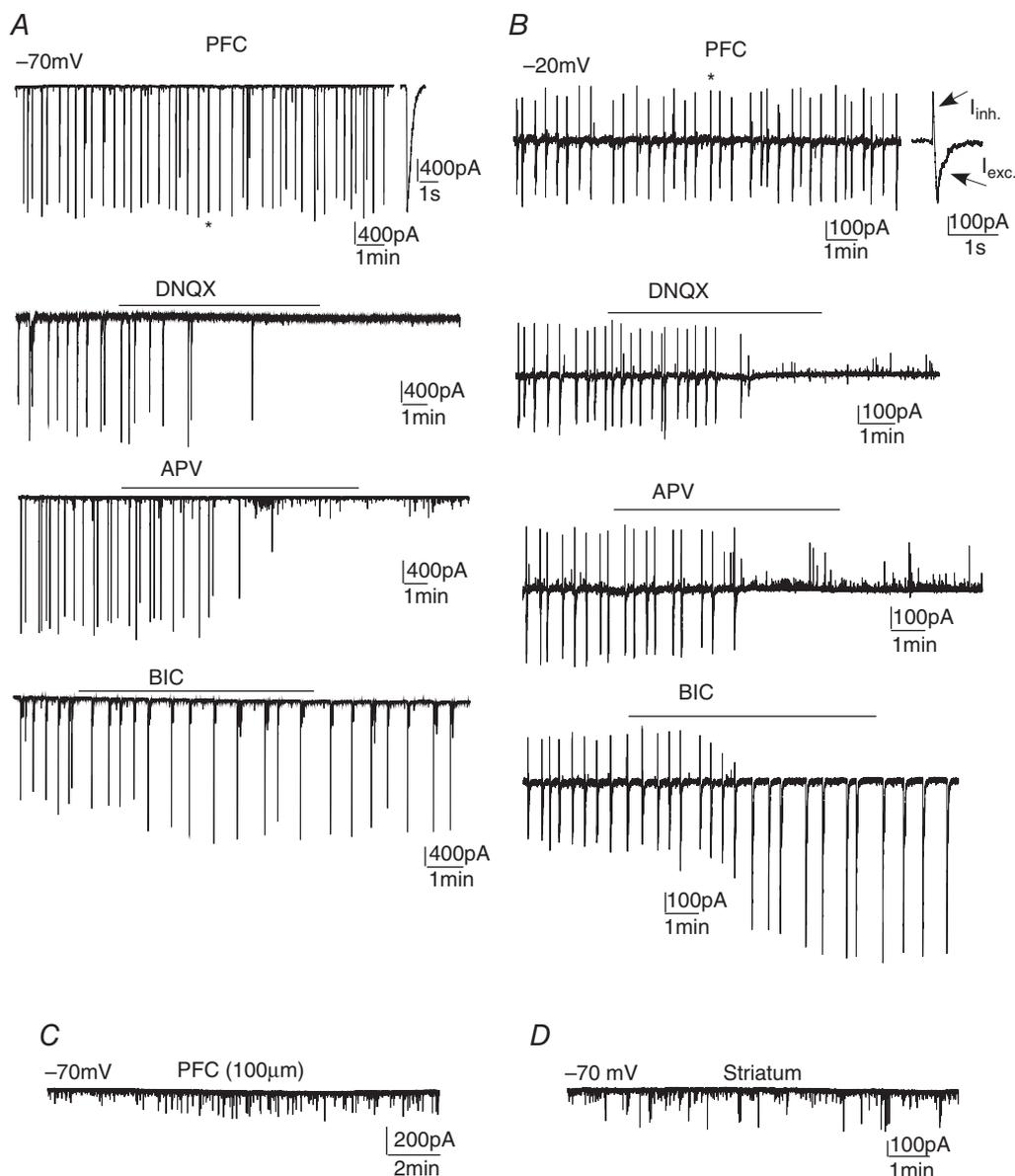


Figure 1. The synchronous network activity in the PFC is dependent on AMPAR- and NMDAR-mediated glutamatergic transmission

Network bursts recorded in PFC pyramidal neurons held at -70 mV (A) or -20 mV (B) in the absence or presence of DNQX (AMPA antagonist), APV (NMDAR antagonist) or bicuculline (GABA_AR antagonist). *Enlarged view of the network bursting-associated currents. At -70 mV, the network burst is made of an inward excitatory current. At -20 mV, the network burst is composed of inward excitatory and outward inhibitory currents. C and D, synaptic currents recorded in PFC pyramidal neurons (held at -70 mV) from thin slices ($100 \mu\text{m}$) or medium spiny neurons (held at -70 mV) from striatal slices.

To reveal inhibitory synaptic currents in the measurement of network activity, we held the membrane potential at a more depolarized level (-20 mV). Under this condition, the rhythmic synchronous network bursting-associated current was composed of two parts: an inhibitory outward current (amplitude: 218 ± 17 pA, $n = 7$) and an excitatory inward current (amplitude: 307 ± 21 pA, $n = 7$) (Fig. 1B). The frequency of

synchronous network activity was not influenced by holding potentials (interval: 19.4 ± 2.1 s, $n = 12$). Application of DNQX ($50 \mu\text{M}$) or APV ($50 \mu\text{M}$) blocked both excitatory inward network current and inhibitory outward network current ($n = 6$) (Fig. 1B). Application of bicuculline blocked the inhibitory outward network current, and dramatically altered the excitatory inward network current (amplitude: $116 \pm 14\%$ increase;

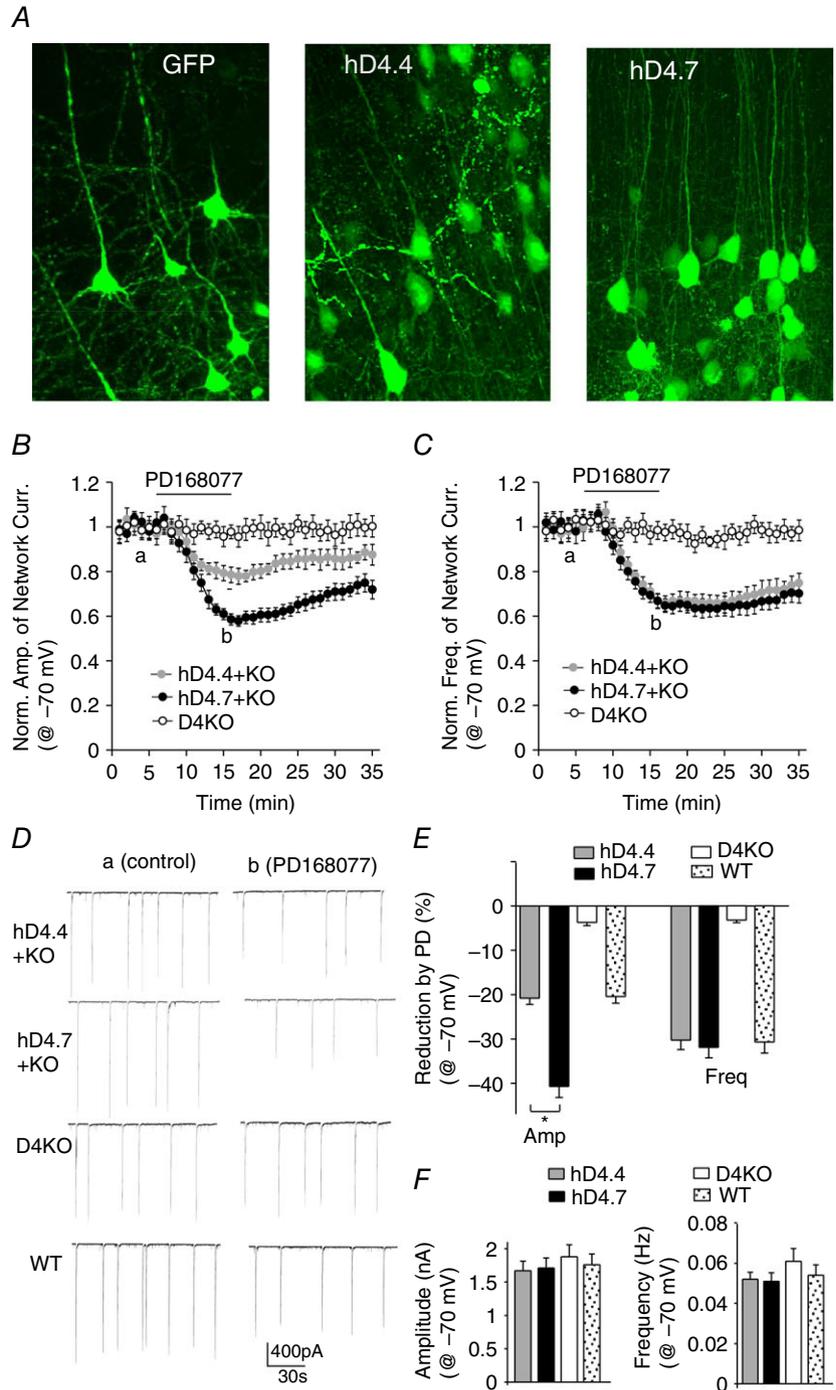


Figure 2. Activation of hD4.7 induces a stronger suppression of PFC synchronous network activity than hD4.4
 A, confocal images of PFC slices from D₄R knockout mice with a stereotaxical injection of GFP or GFP-conjugated hD4.4 or hD4.7 Sindbis virus. Network burst amplitudes (B) and frequencies (C) show the effect of bath applied D₄R agonist PD168077 ($20 \mu\text{M}$) in PFC pyramidal neurons (held at -70 mV) of D₄R knockout mice with or without viral infection of human D4.4 or D4.7 variants. D, representative network bursts in the absence or presence of PD168077 in PFC pyramidal neurons from different groups. E, percentage reduction of the amplitude and frequency of network bursts by PD168077 in different groups. * $P < 0.01$, ANOVA, hD4.4 vs. hD4.7. F, amplitude or frequency of baseline network bursting currents (held at -70 mV) in different groups.

frequency: $46 \pm 5\%$ decrease, $n = 8$). This suggests that glutamatergic transmission, which is mediated by AMPA and NMDA receptors, is required for synchronous network activity, whereas GABAergic transmission modulates oscillatory network activity in the PFC.

Thin PFC slices ($120 \mu\text{m}$), which had severe loss of neuronal circuit integrity, failed to produce the large scale rhythmic synchronous network bursts ($n = 8$) (Fig. 1C). No giant network bursts were observed in medium spiny neurons from striatal slices ($n = 9$) (Fig. 1D). This suggests that the synchronized network activity requires intact neuronal connections in cortical circuits and occurs in a brain region-specific manner.

The effect of hD4.7 and hD4.4 on synchronous network activity in PFC neurons

To clarify the role of human D_4R variants in the cortical network, we examined the effects of the specific D_4R agonist PD168077 on spontaneous network activity in the PFC of D_4R knockout mice with viral infection of hD4.4 or hD4.7. The rodent D_4R only contains two repeats, and wild-type mice were also used for comparison. To avoid high overexpression of the hD4.x Sindbis virus, mice were killed within 2 days after viral delivery. PFC neurons expressing the GFP-conjugated hD4.4 or hD4.7 exhibited normal morphological structures (Fig. 2A). In hD4.7-expressing PFC pyramidal neurons (held at

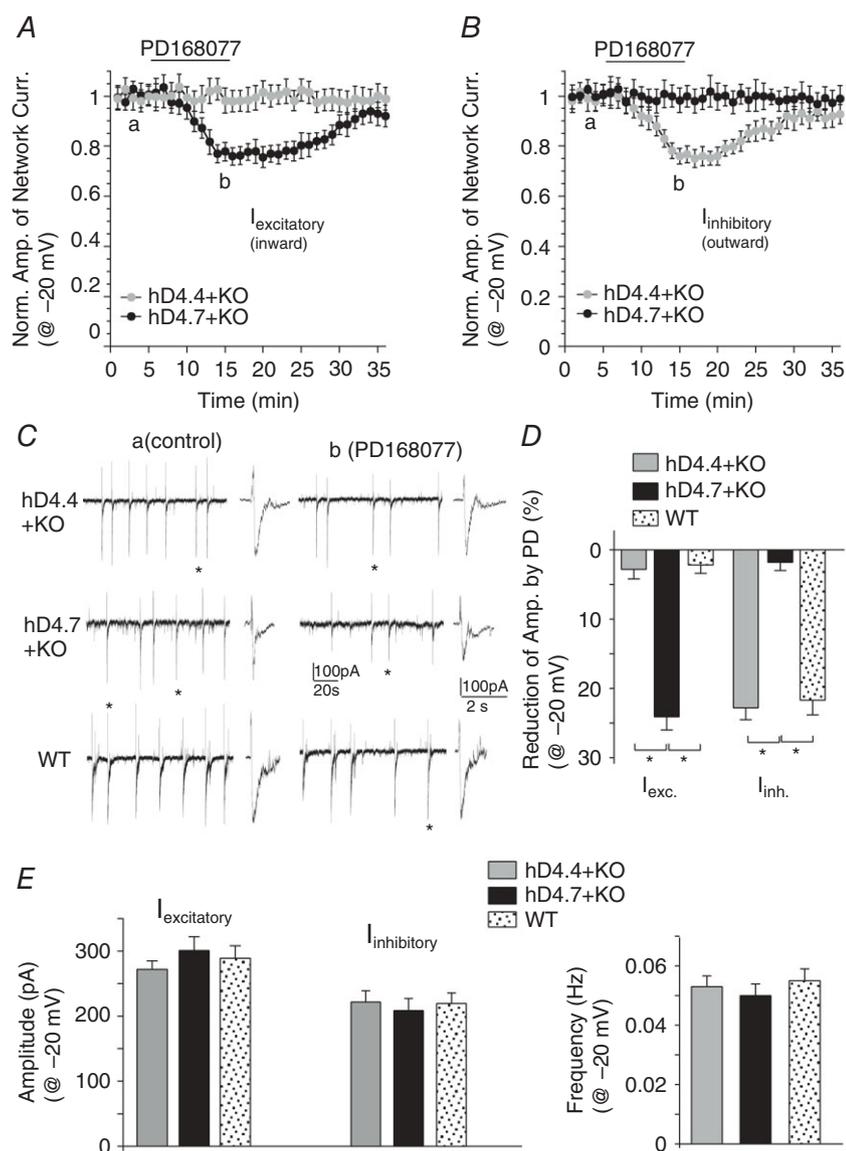


Figure 3. Activation of hD4.7 induces an over-suppression of excitatory network activity and an under-suppression of inhibitory network activity in the PFC

Excitatory or inhibitory network burst amplitudes (A) and frequencies (B) showing the effect of PD168077 ($20 \mu\text{M}$) in PFC pyramidal neurons (held at -20 mV) of D_4R knockout mice with viral infection of human $D_4.4$ or $D_4.7$ variants. C, representative excitatory and inhibitory network bursts in the absence or presence of PD168077 in PFC pyramidal neurons from different groups. D, percentage reduction of the amplitude of excitatory or inhibitory network bursts by PD168077 in different groups. $*P < 0.01$, ANOVA. E, amplitude or frequency of baseline excitatory and inhibitory network bursts (held at -20 mV) in different groups.

−70 mV), PD168077 (20 μM) decreased the amplitude of network bursts by $40.7 \pm 2.5\%$ ($n = 7$), which was significantly ($P < 0.01$) larger than the reducing effect of PD168077 in hD4.4-expressing PFC pyramidal neurons ($20.8 \pm 1.4\%$, $n = 7$) (Fig. 2B, D and E) or in PFC pyramidal neurons from wild-type mice that endogenously express D4.2 ($20.4 \pm 1.5\%$, $n = 6$) (Fig. 2D and E). PD168077 reduced the frequency of network bursts to a similar level in D4.x-expressing neurons (hD4.7: $31.9 \pm 2.4\%$, $n = 7$; hD4.4: $30.3 \pm 2.1\%$, $n = 7$) (Fig. 2C, D and E) and neurons from wild-type mice ($30.7 \pm 2.5\%$, $n = 6$) (Fig. 2D and E). PD168077 had no effect on network activity in D₄R knockout mice (amp: $3.7 \pm 0.7\%$, freq: $3.2 \pm 0.6\%$, $n = 5$) (Fig. 2D and E), confirming the mediation by D₄Rs. The baseline network bursts (held at −70 mV) were similar among different groups (wild-type: 1760 ± 159 pA, 0.054 ± 0.005 Hz; D₄KO: 1880 ± 181 pA, 0.061 ± 0.006 Hz; hD4.4: 1670 ± 145 pA, 0.052 ± 0.004 Hz; hD4.7: 1710 ± 152 pA, 0.051 ± 0.004 Hz) (Fig. 2F). This suggests that hD₄R activation results in a sustained decrease of burst occurrence without disrupting the patterned nature of activity, and activation of the ADHD-linked variant hD4.7 results in more prominent hypoexcitability of the PFC network.

The recordings described above were performed at room temperature (23°C). We also examined the network bursting current and its regulation by D₄R activation at a more physiological temperature (32°C). We found that the rise in temperature increased the network current amplitude and frequency (amplitude: $28.9 \pm 2.2\%$, frequency: $23.6 \pm 2.7\%$, $n = 5$), although the reducing effect of PD168077 on network bursts was similar at different temperatures (23°C, amplitude: $20 \pm 1.5\%$,

frequency: $30.7 \pm 2.5\%$, $n = 6$; 32°C, amplitude: $20.6 \pm 1.9\%$, frequency: $28.8 \pm 2.6\%$, $n = 5$).

Next, we compared the effect of hD4.x on excitatory and inhibitory network bursts in PFC pyramidal neurons held at a depolarized level (−20 mV). In hD4.7-expressing neurons, PD168077 (20 μM) decreased the excitatory inward network current amplitude by $24.1 \pm 1.9\%$ ($n = 8$) (Fig. 3A, C and D), whereas it had no effect on the inhibitory outward network current amplitude ($1.8 \pm 1.2\%$, $n = 8$) (Fig. 3B to D). By contrast, in hD4.4-expressing neurons, PD168077 (20 μM) had no effect on the excitatory inward network current amplitude ($2.8 \pm 1.4\%$, $n = 7$) (Fig. 3A, C and D) but decreased the inhibitory outward network current amplitude by $22.8 \pm 1.7\%$ ($n = 7$) (Fig. 3B to D), which was similar to the effect of PD168077 in the PFC pyramidal neurons from wild-type mice (inward-amplitude: $0.8 \pm 1.6\%$, outward-amplitude: $21.7 \pm 2.1\%$ reduction) (Fig. 3C and D). The reducing effects of PD168077 on network burst frequency were similar in D4.x-expressing neurons (hD4.7: $32.6 \pm 3.5\%$, $n = 8$; hD4.4: $30.3 \pm 2.8\%$, $n = 7$) and those from wild-type mice ($31.1 \pm 2.9\%$, $n = 5$). The baseline network bursts (held at −20 mV) were similar among different groups (wild-type, inward: 289 ± 19 pA, outward: 203 ± 15 pA, 0.055 ± 0.004 Hz; hD4.4, inward: 272 ± 13 pA, outward: 205 ± 16 pA, 0.053 ± 0.004 Hz; hD4.7, inward: 301 ± 21 pA, outward: 193 ± 17 pA, 0.050 ± 0.004 Hz) (Fig. 3E). This suggests that, compared to the normal variant hD4.4, the ADHD-linked variant hD4.7 induces more suppression of glutamatergic excitatory transmission and less suppression of GABAergic inhibitory transmission in the synaptic circuitries, which may collectively result in PFC network hypoactivity.

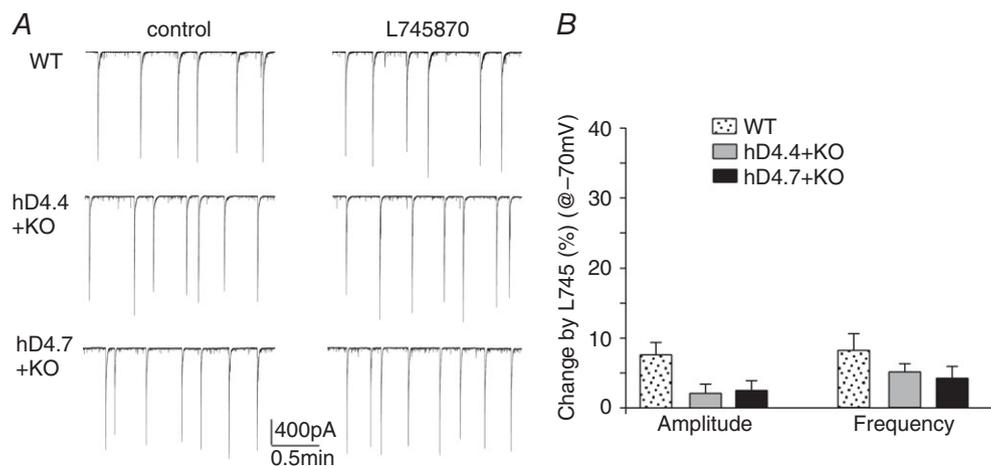


Figure 4. Application of a D4R antagonist does not change network bursting currents

A, representative network bursts (−70 mV) in the absence or presence of the D₄R antagonist L-745870 (20 μM) in PFC pyramidal neurons from a wild-type mouse or D₄R knockout mice with viral infection of human D4.4 or D4.7 variants. B, effect of L-745870 on the amplitude and frequency of network bursts.

To determine whether the virally expressed hD₄R variants are activated by spontaneous dopamine release, we examined the effect of a D₄R antagonist on network activity. As shown in Fig. 4A and B, blocking D₄R with the antagonist L-745870 (20 μ M) did not significantly alter network bursting currents in hD₄.x-expressing neurons (wild-type, amplitude: $7.6 \pm 1.8\%$, frequency: $8.2 \pm 2.4\%$, $n = 6$; hD₄.4, amplitude: $2.1 \pm 1.3\%$, frequency: $5.1 \pm 1.2\%$, $n = 5$; hD₄.7, amplitude: $2.5 \pm 1.4\%$, frequency: $4.2 \pm 1.7\%$, $n = 5$, $P > 0.05$, ANOVA), suggesting that hD₄R variants are not constitutively active.

The normalization of hD₄.7 regulation of PFC network activity by methylphenidate (MPH)

To determine whether the aberrant regulation of PFC network activity by hD₄.7 is related to its role in ADHD, we examined whether MPH, an effective agent for ADHD treatment, could normalize the effects of hD₄.7 on synchronous network bursts in PFC pyramidal neurons. MPH (0.5 mg kg⁻¹) or saline control was i.p. injected to hD₄.7-infected D₄R knockout mice and, 1 h later, animals were killed before slicing (Cheng *et al.* 2014).

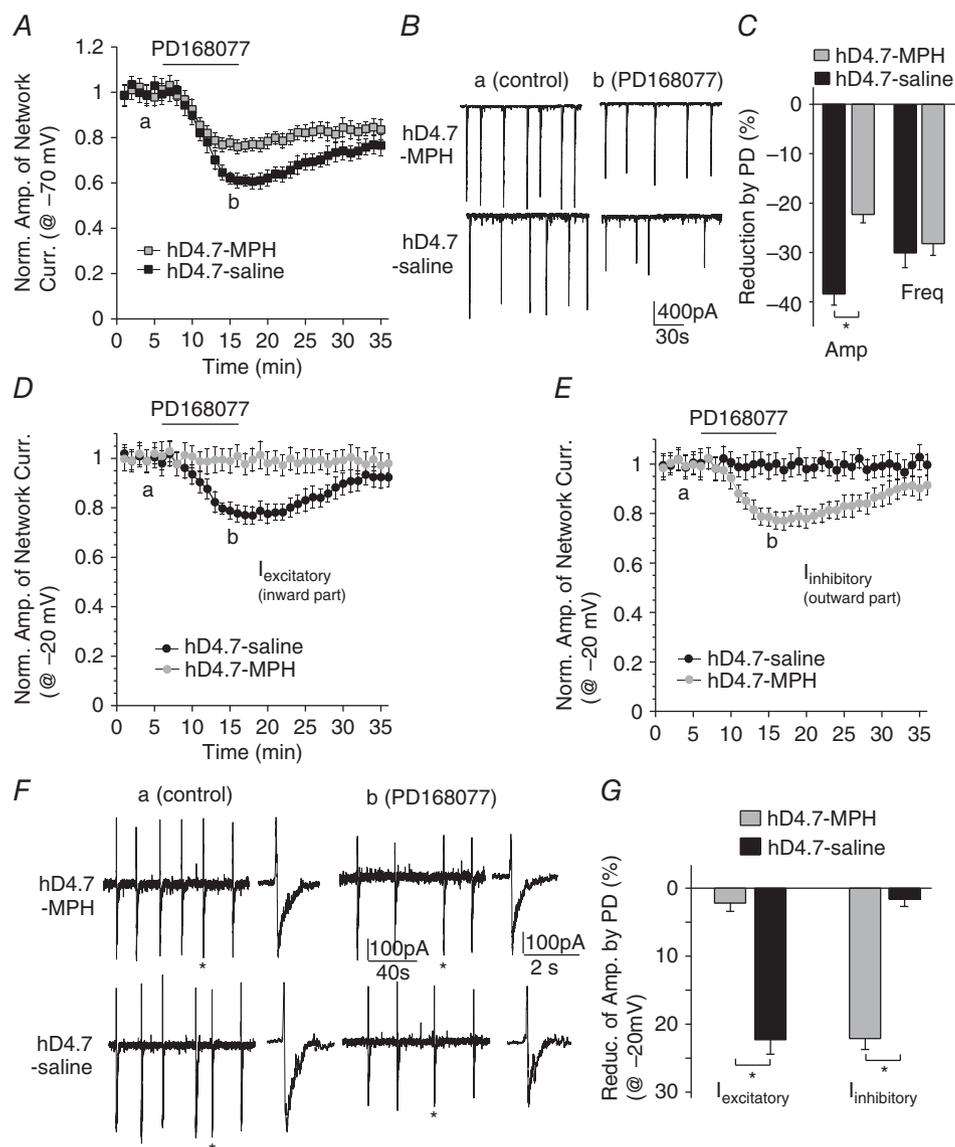


Figure 5. The hD₄.7 regulation of PFC network activity is normalized by MPH injection

A, D and E, network burst amplitudes showing the effect of PD168077 (20 μ M) in PFC pyramidal neurons of hD₄.7-infected, D₄R knockout mice injected with MPH or saline control. B and F, representative network bursts in the absence or presence of PD168077 in hD₄.7-expressing neurons with MPH or saline injection. C and G, percentage reduction of the amplitude of network bursts by hD₄.7 activation in PFC neurons from MPH- or saline-injected mice. Cell membranes were held at -70 mV (A to C) or -20 mV (D to G). * $P < 0.01$, t test, hD₄.7-MPH vs. hD₄.7-saline.

As shown in Fig. 5A to C, in hD4.7-expressing PFC pyramidal neurons (held at -70 mV) from MPH-injected animals, PD168077 ($20 \mu\text{M}$) induced a significantly smaller reducing effect on network burst amplitude than in neurons from saline-injected mice (MPH: $22.3 \pm 1.7\%$, $n = 6$; saline: $38.4 \pm 2.2\%$, $n = 4$, $P < 0.01$). Moreover, in hD4.7-expressing PFC pyramidal neurons (held at -20 mV) from MPH-injected animals (Fig. 5D to G), PD168077 had no effect on the excitatory inward network current amplitude (MPH: $2.2 \pm 1.2\%$, $n = 5$; saline: $22.3 \pm 2.1\%$, $n = 4$), whereas it decreased the inhibitory outward network current amplitude (MPH: $22.1 \pm 1.7\%$, $n = 5$; saline: $1.6 \pm 1.1\%$, $n = 4$). The reducing effect of PD168077 on network current frequency was not affected by MPH (-70 mV, MPH: $30.7 \pm 2.6\%$, $n = 6$; saline: $32.7 \pm 3.2\%$, $n = 4$; -20 mV, MPH: $31.1 \pm 2.9\%$, $n = 5$; saline: $29.4 \pm 2.7\%$, $n = 4$). These data suggest that *in vivo* administration of MPH switches the regulatory effects of hD4.7 on PFC network activity to levels similar to those of hD4.4 (Figs 2 and 3). *In vitro* application of MPH ($20 \mu\text{M}$) decreased network burst amplitude ($26.7 \pm 2.9\%$, $n = 6$) but increased network burst frequency ($31.3 \pm 4.1\%$, $n = 6$) in hD4.7-expressing neurons.

We further examined the impact of MPH in hD4.4-expressing neurons. Compared to saline-injected

mice, MPH injection (i.p.) did not alter the effect of PD168077 on network activity in D4.4-expressing PFC pyramidal neurons recorded at -70 mV (MPH, amplitude: $19.8 \pm 1.5\%$ reduction, frequency: $29.3 \pm 2.3\%$ reduction, $n = 6$; saline, amplitude: $20.9 \pm 1.6\%$ reduction, frequency: $30.9 \pm 2.4\%$ reduction, $n = 6$, Fig. 6A and C) or at -20 mV (MPH, inward-amplitude: $2.8 \pm 0.7\%$, outward-amplitude: $22.9 \pm 1.4\%$ reduction, frequency, $34.5 \pm 3.1\%$ reduction, $n = 6$; saline, inward-amplitude: $-3.7 \pm 1.5\%$, outward-amplitude: $20.1 \pm 1.7\%$ reduction, frequency, $33.3 \pm 2.3\%$, $n = 5$) (Fig. 6B and D).

Discussion

A unique primate-specific feature of D₄R is the additional 2–11 proline-rich repeats located in the third intracellular loop (Wang *et al.* 2004), which allows more complex simultaneous interactions with other proteins containing the SH3 domain. The hD₄R variants with long repeats have been linked to deficiencies in executive control processes in ADHD (LaHoste *et al.* 1996; Swanson *et al.* 1998; Talkowski *et al.* 2008; Gizer *et al.* 2009; Barnes *et al.* 2011). To understand the potential mechanism, we have examined their impact on synchronized network bursts

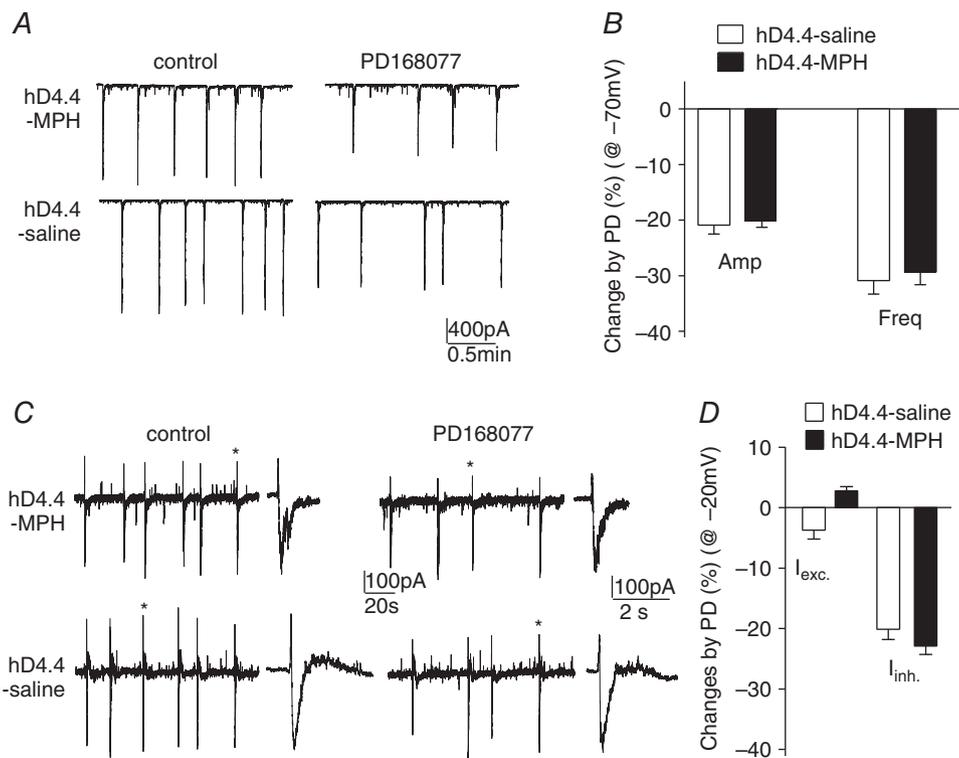


Figure 6. The hD4.4 regulation of PFC network activity is not altered by MPH injection

A and C, representative network bursts in the absence or presence of PD168077 in hD4.4-expressing neurons with MPH or saline injection. B and D, percentage changes of the amplitude or frequency of network bursts by hD4.4 activation in PFC neurons from MPH- or saline-injected mice. Cell membranes were held at -70 mV (A and B) or -20 mV (C and D).

originating from the large scale correlated activity of interconnected neurons, which controls PFC-mediated cognitive function, such as attention (Buschman & Miller, 2007; Miller & Buschman, 2013).

Synchronized network activity in cortex has been suggested to co-operatively support temporal representation and long-term consolidation of information (Buzsáki & Draguhn, 2004). Local and long-range rhythmic synchronization determines neuronal interactions and selective attention (Womelsdorf & Fries, 2007; Womelsdorf *et al.* 2007). Spontaneous synchrony in neural network is expected to depend on the finely tuned interplay of excitatory and inhibitory neuronal populations, which gives rise to the precisely timed and dynamically balanced excitatory and inhibitory conductances (Traub *et al.* 1989). The data obtained in the present study (Fig. 1) indicate that the synchronous network activity requires both AMPA and NMDA receptors, whereas GABAergic transmission mainly modulates oscillatory network activity.

Abnormal synchrony of spontaneous network activity has been associated with various neurological and psychiatric disorders, ranging from epileptic seizures (Steriade, 2003; Garcia Dominguez *et al.* 2005) and Parkinson's disease (Boraud *et al.* 2005; Uhlhaas & Singer, 2006) to schizophrenia (Spencer *et al.* 2003; Uhlhaas & Singer, 2010) and autism (Wilson *et al.* 2007; Yizhar *et al.* 2011). An imbalance favouring excitation may underlie the excessive synchrony in epilepsy (Dichter & Ayala, 1987). The data obtained in the present study (Figs 2 and 3) indicate that activation of hD4.4 reduced excitatory and inhibitory network activity, similar to the effects of D₄ receptor activation in wild-type mice. However, activation of the ADHD-linked hD4.7 induces more suppression of the excitatory network bursts and less suppression of the inhibitory network bursts in the PFC circuitry, suggesting that it shifts the neuronal excitation–inhibition balance towards inhibition in postsynaptic neurons, which may explain the significant frontal hypoactivity detected in ADHD patients (Dickstein *et al.* 2006; Fernández *et al.* 2009).

Little is known about the mechanisms underlying the functional differences of hD₄R variants. Using transfected cell lines, no major discrepancies in pharmacological profiles or the abilities to block cAMP production have been found among hD4.x isoforms (Asghari *et al.* 1995; Jovanovic *et al.* 1999). Using knock-in mice that carry hD4.7 in the third intracellular loop of D₄R, it was found that D4.7 does not form functional heteromers with the dopamine D2S receptor, which is assumed to affect presynaptic dopaminergic control of corticostriatal glutamate release (Gonzalez *et al.* 2012). Our previous studies in rodents have found that activation of D₄ receptors in PFC pyramidal neurons produces a significant reduction of NMDAR-mediated currents (Wang *et al.*,

2003, 2006) and an activity-dependent, homeostatic regulation of AMPAR-mediated transmission (Yuen *et al.* 2010; Yuen & Yan, 2011) via mechanisms dependent on CaMKII and receptor channel trafficking. In addition, rodent D₄R activation in PFC pyramidal neurons leads to a significant decrease of GABA_AR currents (Wang *et al.* 2002) and GABA_AR membrane trafficking via an actin/cofilin/myosin-dependent mechanism (Graziane *et al.* 2009). These effects are similar to those of hD4.4 (normal variant) on excitatory and inhibitory network bursts. The additional proline-rich repeats on hD4.7 (ADHD-linked variant) enable the binding of more proteins containing the SH3 domain, such as many scaffolding proteins in the postsynaptic density (PSD). The binding of dopamine to hD4.7 may change the receptor conformation, disrupting the binding of hD4.7 to these SH3-containing PSD proteins. Thus, the stronger suppression of glutamatergic transmission-mediated excitatory network bursts by hD4.7 activation may be attributable to the larger inhibitory effect of hD4.7 on the membrane trafficking or maintenance of AMPA and NMDA receptors at PSD. Moreover, hD4.7 lost the regulation of GABA_AR-mediated inhibitory network bursts, which may be a result of the attenuated regulation of actin dynamics and the myosin-based transport of GABA_ARs to the surface. The detailed mechanisms await further investigation.

To determine whether the aberrant regulation of PFC network activity by hD4.7 is related to its role in ADHD, we further examined whether MPH, a dopamine reuptake inhibitor approved for ADHD treatment, could normalize the effects of hD4.7 on synchronous network bursts in PFC pyramidal neurons. The data obtained in the present study (Fig. 5) indicate that MPH reduced the effect of hD4.7 on excitatory network bursts, and restored the effect of hD4.7 on inhibitory network bursts, bringing it close to hD4.4 in the regulation of PFC synchronized network activity. The mechanisms underlying MPH-induced changes in hD4.7 regulation of network activity await clarification. In sum, the MPH-induced normalization of synaptic circuitry regulation could contribute to its effectiveness in ADHD treatment.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

ZY and PZ conceived and designed the experiments. PZ and WL collected, analysed and interpreted the data. ZY drafted the article and revised it critically for important intellectual content. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons

designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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