GABAERGIC SYNAPTIC TRANSMISSION ONTO STRIATAL CHOLINERGIC INTERNEURONS IN DOPAMINE D2 RECEPTOR KNOCK-OUT MICE

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Running title: Striatal GABAergic synapse in D2R KO mice

Abstract

Whole-cell or cell-attached analysis was carried out in dopamine (DA) D2 receptor (D2R) knock-out (KO) mice to elucidate the function of this receptor in the regulation of GABAergic synaptic transmission onto striatal cholinergic interneurons as well as their spontaneous firing. In slice preparation obtained from wild type mice, evoked GABAergic inhibitory postsynaptic currents (IPSCs) showed frequency-dependent suppression, and this suppression significantly decreased in the presence of a D2-like receptor antagonist or in D2R KO mice. Contribution of N-type calcium channel was also significantly reduced in the striatal cholinergic interneurons of the D2R KO mice compared with that in the wild type mice. Spontaneous firing of striatal cholinergic interneurons was inhibited by 5 or 10 Hz stimulation, and the suppression was decreased in the presence of a D2-like receptor antagonist or in D2R KO mice. These findings substantiate the physiological role of D2R in the regulation of GABAergic synaptic transmission onto striatal cholinergic interneurons as well as their excitability, confirming the tight coupling between D2R and N-type calcium channels in the regulation of GABA release.

Key words: dopamine receptors, GABAergic synapse, knock-out mice, striatum, cholinergic neuron

INTRODUCTION

Dopaminergic neurons in the substantia nigra pars compacta (SNc) send their axons to medium spiny neurons as well as cholinergic interneurons in the striatum (Freund *et al.*, 1984; Kubota *et al.*, 1987), regulating neuronal activities of these striatal neurons. Nigro-striatal dopaminergic pathway plays important roles in motor control (for review, Kreitzer and Malenka, 2008) through the interaction between dopamine (DA) and acetylcholine (ACh) (for review, Crag, 2006; Pisani *et al.*, 2007). One of the potential neurophysiological events contributing to the motor control is synaptic transmission in the striatum (for review, Calabresi *et al.*, 2007). However, little information has been available on the DA receptor subtypes contributing to the synaptic transmission. In cellular levels, the roles of dopamine and/or DA receptors in striatal medium spiny neurons are still multifaceted; both excitatory and inhibitory effects have been reported (for review, Surmeier *et al.*, 2007). On the other hand, in cholinergic interneurons, activation of postsynaptic D1-like receptors depolarizes the membrane by closing potassium channels or opening non-selective cation channels (Aosaki *et al.*, 1998), whereas activation of presynaptic D2-like receptors located on GABAergic terminals inhibits GABA release onto cholinergic interneurons (Pisani *et al.*, 2000; Momiyama and Koga, 2001) by selectively blocking N-type calcium channels (Momiyama and Koga, 2001). These previous findings raise the question regarding the role of physiologically released DA, as well as on the physiological linkage between DA receptors

and N-type calcium channels.

Using the D2R knock out (D2R KO) mice, we examined (1) the effect of stimulus frequency on GABAergic transmission onto striatal cholinergic interneurons as well as on their spontaneous firing in order to determine the physiological role of endogenously released DA, and (2) the physiological linkage between dopamine D2 receptors (D2R) and N-type calcium channels in the modulation GABA release.

EXPERIMENTAL PROCEDURES

Animal handling and all experimental procedures were done according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences of the Physiological Society of Japan (2009), the UK Animals (Scientific Procedures) Act 1986, and the guidelines of the National Institutes of Natural Sciences, Jikei University School of Medicine and Kitasato University. These procedures were approved by the Animal Care and Use Committee of the National Institutes of Natural Sciences, Jikei University School of Medicine and Kitasato University School of Medicine.

Dopamine receptor deficient mice

Mice lacking D2R were generated according to the protocol by Katsuki and colleagues (Yamaguchi *et al.*, 1996) and backcrossed for up to ten generations with C57BL/6J mice.

Genotypes were determined by PCR analysis of genomic DNA extracted from each mouse's tail. Mice were kept in stable conditions of temperature 22 ± 2 °C and humidity 55 ± 5 % with a 12 hr light-dark cycle.

Slice preparation

Mice (postnatal 20-23 or 35-44 days) were decapitated under deep halothane anesthesia, and coronal slices containing the striatum were cut (300 μ m thick) using a microslicer (PRO7, Dosaka, Kyoto, Japan) in ice-cold oxygenated cutting Krebs solution of the following composition (mM): choline chloride, 120; KCl, 2.5; CaCl₂, 0.5; MgCl₂, 7; NaH₂PO₄, 1.25; NaHCO₃, 26; D-glucose, 15; ascorbic acid, 1.3. The slices were then transferred to a holding chamber containing standard Krebs solution of the following composition (mM): NaCl, 124; KCl, 3; CaCl₂, 2.4; MgCl₂, 1.2; NaH₂PO₄, 1; NaHCO₃, 26; D-glucose, 10; pH 7.4 when bubbled with 95% O₂-5% CO₂. Slices were incubated in the holding chamber at room temperature (21-26 °C) for at least 1 h before recording.

Recording and data analysis

For recording, a slice was transferred to the recording chamber, held submerged, and superfused with standard Krebs solution (bubbled with 95% O₂-5% CO₂) at a rate of 3-4 ml min⁻¹. Neurons within the striatum were visually identified with a 60x water immersion

objective attached to an upright microscope (BX50WI, Olympus Optics, Tokyo, Japan). Images were detected with a cooled CCD camera (CCD-300T-RC, Nippon Roper, Japan) and displayed on a video monitor (LC-150M1, SHARP, Japan). Pipettes for whole-cell recordings were made from standard-walled borosilicate glass capillaries (1.5 mm outer diameter; Clark Electromedical, Reading, UK). For synaptic currents recordings, patch pipettes were filled with a CsCl-based internal solution of the following composition (mM): CsCl, 140; NaCl, 9; Cs-EGTA, 1; Cs-HEPES, 10, Mg-ATP, 2 (pH adjusted with 1M CsOH). For current-clamp recordings, a K-gluconate-based internal solution of the following composition (mM) was used: K-gluconate, 120; NaCl, 6; CaCl₂, 5; MgCl₂, 2; K-EGTA, 0.2; K-HEPES, 10; Mg-ATP, 2; Na-GTP, 0.3 (pH adjusted with 1 M KOH). Whole-cell recordings were made from visually identified cholinergic interneurons within the striatum using a patch-clamp amplifier (Axopatch 200B, Molecular Devices, Foster City, CA, USA). The series resistance was measured from the amplifier, and the data were discarded, if the series resistance changed by more than 10 % of the initial value. The access resistance was monitored by measuring capacitative transients obtained in response to a hyperpolarizing voltage step (5 mV, 25 ms) from a holding potential of -60 mV. No correction was made for the liquid junction potentials (calculated to be 5.0 mV by pCLAMP7 software, Molecular Devices). Synaptic currents were evoked by extracellularly delivered voltage pulses (0.2-0.4 ms in duration) of suprathreshold intensity via a stimulating electrode filled with 1 M NaCl. The stimulating electrode was

placed within 50-120 μm radius of the recorded neuron. The position of the stimulating electrode was varied until a stable response was evoked in the recorded neuron. All the inhibitory postsynaptic currents (IPSCs) were evoked at a holding potential of -60 mV . Experiments were carried out at room temperature ($21\text{-}26^{\circ}\text{C}$).

Data were stored on digital-audio tapes using a DAT recorder (SONY, Japan, DC to 10 kHz). Synaptic currents were digitized off-line at 10 kHz (low-pass filtered at 2 kHz with an 8-pole Bessel filter) using pCLAMP9 software (Molecular Devices). The effects of drugs on the evoked IPSCs were assessed by averaging their amplitudes for 100 s (20 traces) after the effect had reached the steady state and comparing this value with the averaged amplitude of 20 traces just before the drug application. Statistical analysis was carried out using both Student's *t* test (two-tailed) and a non-parametrical Mann-Whitney *U* test. P value of 0.05 was used as the confidence limit and data are expressed as mean $\pm$ S.E.M.

Drugs

Synthetic blocker of N-type calcium channel, ω -conotoxin GVIA (ω -CgTX), and synthetic blocker of P/Q-type calcium channel, ω -agatoxin TK (ω -Aga-TK) were purchased from Alamone labs (Jerusalem, Israel). These toxins were dissolved in oxygenated perfusing solution containing cytochrome *c* (1 mg ml^{-1} , Sigma). Other drugs were stored as frozen stock solutions and dissolved in the perfusing solution just before application in the final

concentration indicated. Bicuculline methochloride, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) from Tocris Cookson (Bristol, UK). Trans-(-)- 4aR- 4,4a,5,6,7,8,8a,9- octahydro-5- propyl-1H-pyrazolo[3,4-g]quinoline hydrochloride (quinpirole) and S(-)-5-aminosulfonyl -N-[(1-ethyl-2-pyrrolidiny)-methyl]-2-methoxybenzamide (sulpiride) were from Research Biochemicals International (Natic, MA). Strychnine was from Sigma (St Louis, MO, USA). All drugs were bath applied.

RESULTS

Identification of striatal interneurons in mice

Whole-cell recordings were made from 82 (45 in wild type mice and 37 in D2R KO mice) cholinergic interneurons within the striatum, identified visually and electrophysiologically. Neurons in the striatum were viewed with a microscope fitted with a water-immersion optic (Nikon, Tokyo, Japan or Olympus BX50WI, Olympus, Tokyo, Japan). Only those within the dorsal part of the striatum were used in the present study, similarly to the previous study in rats (Momiya and Koga, 2001). Large neurons (the long axis > 30 μ m, Fig. 1A) were selected for whole-cell recording according to the size distribution of cholinergic and non-cholinergic neurons in the striata of rats (Kawaguchi, 1992) or mice (Govindaiah *et al.*, 2010). In order to functionally identify striatal cholinergic neurons, membrane properties of these large cells were also examined using a K-gluconate-based internal solution (Fig. 1B).

When hyperpolarizing current pulses were applied through the recording pipette containing K⁺-based internal solution under the current-clamp mode, these neurons exhibited a characteristic voltage sag during a large hyperpolarization (Fig. 1B), similarly to the previous studies using rats (Jiang and North, 1991; Kawaguchi, 1992; Kawaguchi *et al.*, 1995; Aosaki *et al.*, 1998) or mice (Govindaiah *et al.*, 2010). The voltage sag was also observed with Cs⁺-based internal solution to block the inwardly-rectifying K⁺ conductance, similarly to the case in rats (Momiya and Koga, 2001) or mice (Govindaiah *et al.*, 2010). Therefore, large neurons that exhibited a characteristic voltage sag during a large hyperpolarization were used for the present study.

GABAergic synaptic transmission

Synaptic currents were evoked at a holding potential of -60 mV by focal stimulation in the presence of CNQX (5 μ M), D-AP5 (25 μ M) and strychnine (0.5 μ M) to block glutamatergic and glycinergic components (Fig. 1C). The evoked currents were reversibly blocked by bath application of bicuculline (10 μ M, Fig. 1C) in all of 6 neurons examined, confirming that they are GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) in nature, similar to our previous study in rats (Momiya and Koga, 2001). Thus in the rest of the study, GABAergic IPSCs were pharmacologically isolated using the same combination of blockers.

IPSC amplitude evoked at 0.2 Hz in wild type and D2R KO mice was -272.6 ± 25.7 pA

(n=41) and -212.8 ± 24.3 pA (n=27), respectively. The difference between these two values was not significantly different ($P>0.05$).

Selective coupling of D2 receptors with N-type calcium channels

In wild type mice, bath application of quinpirole, a dopamine D2-like receptor agonist, at a concentration of 30 μ M inhibited the evoked IPSCs in a reversible manner (Fig. 2A), similarly to the previous studies in rats (Pisani *et al.*, 2000; Momiyama and Koga, 2001). On the other hand, quinpirole had little or no effect on the IPSCs in D2R KO mice (Fig. 2B). Quinpirole-induced inhibition of IPSC amplitude in wild type and D2R KO mice was 32.9 ± 2.24 % (n=4) and 3.28 ± 1.38 % (n=6), respectively (Fig. 2C).

Bath application of an N-type calcium channel blocker, ω -CgTX (3 μ M) caused a gradual decline in the amplitude of IPSCs, reaching a steady-state in 5-10 min in wild type mice (Fig. 2A). The degree of suppression by ω -CgTX was 58.6 ± 1.97 % (n=6, Fig. 5A and C), comparable to that in rats (Momiyama and Koga, 2001). After confirming quinpirole (30 μ M)-induced inhibition had showed complete recovery following washout, ω -CgTX (3 μ M) was applied, and after ω -CgTX's effect had reached a steady-state, quinpirole (30 μ M) was applied again. Quinpirole no longer suppressed IPSCs in the presence of ω -CgTX (2.07 ± 0.53 % inhibition, n=4, Fig. 2A and 2C). These results indicate that the inhibitory action of quinpirole was selectively coupled to presynaptic N-type calcium channels, confirming the

selective coupling between D2-like receptors and N-type calcium channels suggested in our previous study using rats (Momiyama and Koga, 2001).

Frequency-dependent suppression of IPSCs

Inhibition of IPSCs evoked in striatal cholinergic interneurons by exogenously applied quinpirole, a D2-like receptor agonist, raises the question of the modulatory roles of DA physiologically released in the striatum, since dopaminergic axons originated from dopaminergic neurons in the substantia nigra pars compacta terminate in striatal cholinergic interneurons (Kubota *et al.*, 1987). To address the question, dependency of the evoked IPSCs on the stimulus-frequency was examined between 0.2 and 10 Hz. Mean IPSCs were collected once IPSC amplitude had reached steady state during sustained stimulation (Silver *et al.*, 1998). IPSC amplitude at each stimulus frequency was judged by visual inspection that it had reached steady state. At 0.5-2 Hz, the amplitude reached steady state within 1 min. At 3.3, 5, or 10 Hz, it reached steady state within 10-20 s. IPSC amplitude at each stimulus frequency returned to the basal level within several seconds after the stimulus frequency was returned to 0.2 Hz, and the duration of recovery to the basal level could correspond to the duration of D2-like receptor mediated slow synaptic potential evoked in midbrain dopaminergic neurons (Beckstead *et al.*, 2004). IPSCs evoked in striatal cholinergic interneurons of wild type mice showed frequency-dependent suppression during sustained stimulation. This is illustrated in

Figure 3A, which shows averaged IPSCs obtained at various stimulus frequencies (0.2-10 Hz) under steady state conditions. The relationship between IPSC peak amplitude and stimulus frequency is shown in Figure 3B. In these series of experiments, the averaged IPSC amplitude in wild type mice evoked at 0.2 Hz was -266.3 ± 43.3 pA (n=16). The amplitude evoked at 0.5, 1, 2, 3.3, 5 and 10 Hz was 0.80 ± 0.01 (n=16), 0.72 ± 0.03 (n=16), 0.51 ± 0.04 (n=14), 0.50 ± 0.04 (n=6), 0.38 ± 0.02 (n=12) and 0.19 ± 0.01 (n=12) as a fraction of the corresponding neurons' IPSC amplitude at 0.2 Hz, respectively (Fig. 3B, open circles). In order to clarify the receptors involving frequency-dependent suppression of IPSCs, the effect of sulpiride, a D2-like receptor antagonist, was examined on the frequency dependent inhibition of IPSCs in wild type mice. After 10 min perfusion of external solution containing sulpiride at a concentration of 10 μ M, the same frequent-dependency was examined. IPSC amplitude in wild type mice evoked at 0.5, 1, 2, 5 and 10 Hz in the presence of sulpiride was 0.90 ± 0.06 (n=6), 0.79 ± 0.03 (n=6), 0.58 ± 0.02 (n=6), 0.51 ± 0.02 (n=6) and 0.32 ± 0.06 (n=6), respectively (Fig. 3B, open squares). The difference between in the absence and presence of sulpiride was significant ($P < 0.05$) in IPSC amplitude evoked at 5 and 10 Hz. The weak inhibition in the presence of sulpiride was not significant ($P > 0.05$) at lower frequencies. When the effect of sulpiride was compared between the corresponding 6 neurons, the difference was significant ($P < 0.05$) only at 10 Hz.

In terms of specificity for the receptor subtypes, utilization of receptor knock out mice is a

better tool than pharmacological manipulation. Therefore, frequency-dependent suppression of IPSCs was examined in dopamine D2R KO mice. In these series of experiments, the averaged IPSC amplitude in D2R KO mice evoked at 0.2 Hz was -262.9 ± 56.7 pA (n=7). As shown in Figures 3A and 3B, frequency-dependent suppression of IPSCs was reduced in D2R KO mice, and the reduction was apparently larger than that observed in wild type mice with sulpiride. The IPSC amplitude in D2R KO mice at the frequency of 0.5, 1, 2, 3.3, 5 and 10 Hz was 0.99 ± 0.01 (n=7), 0.91 ± 0.02 (n=7), 0.84 ± 0.07 (n=7), 0.87 ± 0.01 (n=5), 0.62 ± 0.06 (n=7) and 0.44 ± 0.05 (n=7), respectively as a fraction of that at 0.2 Hz (Fig. 3B, closed circles). The value in D2R KO mice was significantly ($P < 0.05$) different from that in wild type mice at each of the corresponding stimulus frequency.

High frequency stimulation-induced effect on the firing of cholinergic interneurons

Frequency-dependent suppression and its reduction in D2R KO mice as well as in the presence of sulpiride indicate endogenous release of DA with high frequency stimulation might be involved in this suppression. A previous study has reported D2-like receptor agonist-induced modulation of firing frequency of cholinergic interneurons (Maurice *et al.*, 2004). Therefore, effects of high frequency stimulation (5 and 10 Hz), that mimic the spontaneous firing rate of dopaminergic neurons in the midbrain (Beckstead *et al.*, 2004), on the spontaneous firing of cholinergic interneurons was examined, using cell-attached

recording technique with K-gluconate pipette solution. The site and intensity of stimulation was similar to that in the case of evoked IPSCs.

Spontaneous firing rate with cell-attached recording of cholinergic interneurons in slices obtained from wild type mice and D2R KO mice was 5.67 ± 0.94 Hz (n=6) and 5.13 ± 0.26 Hz (n=6), respectively. Similar stimulating protocol was applied to that used in the suppression of IPSCs; 15 s stimulation at 5 Hz and 10 s stimulation at 10 Hz. Mean firing rate was calculated just before and after the stimulation. In wild type mice, firing rate was reduced to 3.45 ± 0.64 and 2.85 ± 0.57 Hz (each n=6) after 5 and 10 Hz stimulation, respectively (Fig. 4A and C), similar to the case of agonist activation of D2-like receptors (Maurice et al., 2004). Similar to the case of evoked IPSCs, stimulation-induced inhibition of firing was recovered to the basal level within 10 s after the cessation of stimulation. After the recovery of stimulation-induced inhibition of firing, sulpiride, a D2-like receptor antagonist at a concentration of 10 μ M was applied in the bath for 5-10 min, and stimulation was again applied in the presence of sulpiride. Firing rate in the presence of sulpiride was 5.71 ± 1.15 Hz, which was not significantly ($P < 0.05$) from control (5.67 ± 0.94 Hz, n=6). Stimulation induced inhibition of firing was antagonized in the presence of sulpiride. In the presence of sulpiride, the firing rate after 5 and 10 Hz stimulation was 5.34 ± 1.15 Hz (n=6) and 5.21 ± 1.16 Hz (n=6), respectively, significantly ($P < 0.05$) larger than that without sulpiride (Fig. 4A and C). Spontaneous firing rate of cholinergic interneurons in slices from D2R KO mice was

5.13 $\pm$ 0.26 Hz (n=6), which was not significantly different ($P>0.05$) from that of wild type mice (5.67 $\pm$ 0.94 Hz, n=6). In D2R KO mice, 5 and 10 Hz stimulation had no significant ($P>0.05$) effect on the firing rate (Fig. 4B). Firing rate after 5 and 10 Hz stimulation was 5.40 $\pm$ 0.33 Hz (n=6) and 5.49 $\pm$ 0.43 Hz (n=6), respectively, which was not significantly ($P>0.05$) different from the value before stimulation (5.13 $\pm$ 0.26 Hz, n=6, Fig. 4C).

Contribution of calcium channel subtypes

The evident selective coupling between D2-like receptors and N-type calcium channels raises another question how the effect of deletion of D2R is. To address the question, contribution of calcium channel subtypes in the GABAergic transmission onto striatal cholinergic interneurons was examined using D2R KO mice. IPSCs were evoked in striatal cholinergic interneurons by focal stimulation at 0.2 Hz at the holding potential of -60 mV. Figure 5Aa shows the effect of an N-type calcium channel blocker, ω -CgTX (3 μ M) on the amplitude of the evoked IPSCs in a wild type mouse. IPSCs were gradually suppressed by bath application of ω -CgTX and the effect reached the steady state in 5-10 min. IPSCs were inhibited by ω -CgTX by 58.6 $\pm$ 1.97 % (n=6, Fig. 5C) in wild type mice. As shown in Figure 5Aa, additional application of a P/Q-type calcium channel blocker, ω -Aga-TK (200 nM) after ω -CgTX-induced effect had reached steady state further suppressed the IPSCs. The inhibition of IPSCs by additional application of ω -Aga-TK was 96.4 $\pm$ 0.57 % of the control (n=4, not

shown). Figure 5Ba shows the effect of ω -Aga-TK (200 nM) and additional application of ω -CgTX (3 μ M) on the IPSCs. The inhibitory effect of ω -Aga-TK was 76.2 ± 2.96 % (n=7, Fig. 5D) of the control in wild type mice. Additional application of a ω -CgTX after ω -Aga-TK-induced effect had reached steady state suppressed the IPSCs by 98.1 ± 1.40 % (n=7, not shown).

Figure 5Ab shows the effect of ω -CgTX and additional application of ω -Aga-TK on the IPSCs in a D2R KO mouse. Inhibition by ω -CgTX was smaller in D2R KO than in wild type mice. The inhibitory effect of ω -CgTX in these mice was 26.4 ± 5.75 % (n=6, Fig. 5C), which was significantly ($P<0.05$) smaller than the value of wild type mice (58.6 ± 1.97 %, Fig. 5C). On the other hand, the ω -Aga-TK-induced inhibitory effect was significantly larger in D2R KO mice ($87.2. \pm 0.94$ %, n=5) than in wild type mice (76.2 ± 2.96 %, n=7), as shown in Figures 5B and D ($P<0.05$).

Developmental changes in ω -CgTX-induced effect in wild type and D2R KO mice

It has been reported, in the striatum of rats, that ω -CgTX-induced inhibition of IPSCs declines with age in the same GABAergic synapse as in the present study (Momiya, 2003). Therefore, developmental changes were elucidated in ω -CgTX-induced inhibition of IPSCs in D2R KO as well as wild type mice. The present study has so far used wild type and D2R KO mice of postnatal days between 20 and 23 days (P20-23). In the following experiments,

ω -CgTX-induced inhibition of IPSCs was examined using P35-44 mice (adult group, wild type and D2R KO) to compare the values obtained in the above experiments using the young (P20-23) group mice.

In the adult wild type mice, ω -CgTX-induced inhibition of IPSCs was $18.2 \pm 3.21 \%$ ($n=7$, Fig. 5E), that was significantly ($P<0.05$) smaller than that in the young group ($58.6 \pm 1.97 \%$, $n=6$, Fig. 5E). The finding was similar developmental decline to that observed in rats (Momiya, 2003). On the other hand, in D2R KO mice, ω -CgTX-induced inhibition of IPSCs was $26.9 \pm 2.55 \%$ ($n=6$, Fig. 5E), that was not significantly ($P>0.05$) different from that in young D2R KO mice ($26.4 \pm 5.75 \%$, $n=6$, Fig. 5E).

DISCUSSION

By means of D2R KO mice, the present study has revealed a physiological role of D2R in the regulation of GABAergic synaptic transmission onto striatal cholinergic interneurons, as well as the tight coupling between D2R and N-type calcium channels in the regulation of GABA release.

The present study has focused only on inhibitory transmission onto cholinergic interneurons. Although the changes in motor activities may be directly affected by the modulation of medium spiny neurons (MSNs) which express either D1R or D2R,

dopamine-induced effect on the activity of medium spiny neurons reported by previous studies are still ambiguous and multifaceted (Surmeier *et al.*, 2009). On the other hand, dopamine receptor-mediated effects on striatal cholinergic interneurons reported to date have been comprehensive; D5 receptor-mediated enhancement of Zn²⁺-sensitive GABA_A currents (Yan and Surmeier, 1997), D1-like receptor-mediated depolarization of the membrane (Aosaki *et al.*, 1998), presynaptic D2-like receptor-mediated inhibition of GABA release onto cholinergic interneurons (Pisani *et al.*, 2000; Momiyama and Koga. 2001), and D2-receptor-mediated reduction of autonomous activity through modulation of voltage-dependent Na⁺ channels (Maurice *et al.*, 2004). In terms of modulation of firing, agonist activation of D2-like receptors has been reported to reduce firing frequency of most of cholinergic interneurons (Maurice *et al.*, 2004; Wang *et al.*, 2013). Furthermore, receptor subtypes involved in the presynaptic and postsynaptic actions of dopamine has been reported using DA receptor mutant mice (Centonze *et al.*, 2003). In addition, activities of cholinergic interneurons are indirectly affected by dopamine-dependent modulation of MSN activity. For example, substance P released from D1R expressing MSNs has been shown to affect ACh release (Arenas *et al.*, 1991). Therefore, there should be a rationale for relating activities of striatal cholinergic interneurons to motor activities.

The present electrophysiological experiments showed frequency-dependent suppression of IPSCs was present in striatal cholinergic interneurons of wild type mice, and this suppression

was decreased in D2R KO mice. Previous studies have reported DA or quinpirole, a D2-like receptor agonist, inhibits IPSCs by presynaptic mechanisms (Pisani *et al.*, 2000; Momiyama and Koga, 2001). Therefore, the present findings suggest that intrinsic DA activates D2R and thereby inhibits GABA release, demonstrating a physiological role for DA. The manner of termination of dopaminergic axons from substantia nigra pars compacta is quite different from that of GABAergic terminals in the striatum (Freund *et al.*, 1984; Kubota *et al.*, 1987); the former does not form strict synaptic structures observed with fast excitatory or inhibitory synapses. Therefore, DA-induced modulatory effects are expected to be prominent under unusual conditions. Generally, trains of stimuli are required to induce DA-mediated synaptic responses (Beckstead *et al.*, 2004). Frequency-dependent suppression of synaptic currents has been reported at climbing fibre-Purkinje cell synapses, and several mechanisms have been proposed (Silver *et al.*, 1998). The attenuation in frequency-dependent suppression of inhibitory inputs onto cholinergic interneurons observed in D2R KO mice would imply that cholinergic interneurons receive more inhibition overall in D2R KO mice (Fig. 3A and 3B). It remains to be elucidated how this enhanced inhibition of cholinergic interneurons in D2R KO mice results in a hypoactive phenotype (Yamaguchi *et al.*, 1996). Also in the striatal GABAergic synapses onto cholinergic interneurons, there should be unknown mechanisms other than D2-like receptor-mediated inhibition, including those mediated by some other monoamines or peptides.

In the present study, contribution of N-type calcium channels in the GABAergic transmission onto striatal cholinergic interneurons was significantly decreased in D2R KO mice, whereas P/Q-type calcium channel contribution increased. This seems functionally reasonable, since our previous study has shown a selective coupling between D2-like receptors and N-type calcium channels in the modulation of GABA release onto cholinergic interneurons (Momiya and Koga, 2001, Fig.5A). Deletion of D2R could downregulate the coupled downstream effectors. Actually, it has been demonstrated, using co-immunoprecipitation and pull down assays, that D2R and N-type calcium channels form physical signaling complex, and that D2R regulate N-type calcium channel trafficking (Kisilevsky and Zamponi, 2008), suggesting that absence of D2R in D2R KO mice may aberrantly affect the trafficking of N-type calcium channels to the membrane, thereby reducing their contribution to inhibitory transmission. The coupling between D2R and N-type calcium channels has been further suggested in the present study, since the developmental changes was not observed in the contribution of N-type calcium channels in GABAergic transmission onto striatal cholinergic interneurons in D2R KO mice, whereas ω -CgTX-induced inhibition was significantly decreased in adult wild type mice compared to young mice, similar to the results in rats (Momiya, 2003). These findings suggest that such a developmental change could occur only when the coupling partners are normally present in the synapse.

The present findings using cell-attached recording have confirmed the notion that DA could modulate the overall firing of striatal cholinergic neurons through the action on D2-like receptors. In addition, frequency-dependent suppression of IPSCs suggests that D2R located on GABAergic presynaptic terminals could have a considerable role in the regulation of neuronal activity, as suggested by the previous study (Momiyama and Koga, 2001). Although suppression of inhibitory transmission onto striatal cholinergic neurons by the activation of presynaptic D2R could result in the excitation of cholinergic neurons and therefore may lead to the enhanced ACh release in the striatum, the present data using cell-attached recording has shown high frequency stimulus-induced suppression of firing rate, consistent with previous studies showing D2-like receptor agonist-induced inhibition of the firing of striatal cholinergic interneurons by modulation of voltage-dependent Na⁺ channels (Maurice *et al.*, 2004; Wang *et al.*, 2013). It has also been shown that D2R activation leads to a slowing of pacemaker discharge (Maurice *et al.*, 2004). Previous neurochemical studies have also reported a real physiological phenomenon that D2-like receptor agonists reduce ACh release (for review, Di Chiara *et al.*, 1994). There may be other unknown mechanisms that participate in the net modulation of neuronal activities of striatal cholinergic interneurons.

GABAergic inputs to striatal cholinergic neurons are likely to be originating from axon collaterals of striatal medium spiny neurons (Bolam *et al.*, 1986; Martone *et al.*, 1992) or possibly from GABAergic interneurons (Bennett and Wilson, 1998; Koós and Tepper, 1999).

The projection pattern, DA receptor subtypes and neurochemical markers of medium spiny neurons have been reported to be heterogeneous (Gerfen, 2000 and references therein). To date, the quantitative anatomical information for the identification of GABAergic inputs to striatal cholinergic neurons is not yet available. It also remains to be seen the precise subcellular immunocytochemical localization of identified DA receptor subtypes expressed in medium spiny neurons, which could be revealed at the electron microscopy level by the use of specific antibodies for DA receptor subtypes.

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Legends for Figures

Fig. 1. Striatal cholinergic interneurons in wild type mice

A, A striatal cholinergic interneuron in a brain slice obtained from a wild type mouse (postnatal day 20) viewed under IR-DIC microscopy. The recording pipette is attached to the surface of the neuron, and the stimulating electrode is placed close to the recorded neuron. The slice was 300 μm in thickness. **B**, Membrane potentials (above) recorded from another striatal cholinergic interneuron in response to hyperpolarizing current pulses (below) applied intracellularly through Cs^+ -filled electrodes. Voltage sags during hyperpolarizing current pulses, characteristic to striatal cholinergic neurons, were identified. **C**, GABAergic inhibitory postsynaptic currents (IPSCs) recorded in a striatal cholinergic neuron. Each point in the time

course plot represents the amplitude of an individual IPSC evoked at 0.2 Hz (every 5s) in the presence of CNQX (5 μ M), D-AP5 (25 μ M) and strychnine (0.5 μ M) through a Cs⁺-filled electrode. The holding potential was -60 mV. Superimposed traces on the right are averages of 20 consecutive IPSCs during the indicated periods. IPSCs were blocked by bath-applied bicuculline (10 μ M).

Fig. 2. Selective coupling of D2-like receptors with N-type calcium channels

A, Time course of effects of quinpirole (Q, 30 μ M) on IPSCs before and after application of ω -CgTX (3 μ M) in a wild type mouse. Amplitudes of IPSCs evoked at 0.2 Hz are plotted against time at the holding potential of -60 mV with CsCl-based internal solution. Quinpirole-induced inhibition of IPSCs was occluded in the presence of ω -CgTX. Superimposed traces on the right are averages of 20 consecutive IPSCs during the indicated periods. **B**, Lack of quinpirole-induced effect in a D2R KO mouse. Superimposed traces on the right are averages of 20 consecutive IPSCs during the indicated periods. **C**, Summarized histograms from pooled data showing the effects in **A** and **B**. Percent inhibition of IPSCs in wild type mice by quinpirole, quinpirole in the presence of ω -CgTX, and quinpirole in D2R KO mice was, 32.9 ± 2.24 % (n=4), 2.07 ± 0.53 % (n=4), and 3.28 ± 1.38 % (n=6), respectively.

Fig. 3. Frequency-dependent suppression of IPSCs in striatal cholinergic interneurons

A, IPSCs were evoked in striatal cholinergic interneurons in the presence of CNQX (5 μ M), D-AP5 (25 μ M) and strychnine (0.5 μ M) at the holding potential of -60 mV. The patch pipette contained a CsCl-based internal solution. Superimposed traces are averages of 10 consecutive IPSCs evoked in a wild type (WT) mouse (left) and a D2R KO mouse (right). The averages were obtained after the IPSC amplitude had reached the steady state after the stimulus onset of each frequency. Each stimulus frequency is indicated on the left of traces evoked in the wild type mouse. Stimulus artifacts have been omitted. Note the lack of suppression of IPSCs in the D2R KO mouse. **B**, Summarized plots showing the frequency dependent suppression of IPSC as a fraction of amplitude at 0.2 Hz in WT (open circles), WT in the presence of sulpiride (10 μ M, open squares) and D2R KO mice (closed circles). The difference between with and without sulpiride in WT was significant ($P < 0.05$) at 5 and 10 Hz ([#]). The suppression was significantly ($P < 0.05$) reduced in D2R KO mice at each of the corresponding stimulus frequency (*). Each point shows mean with S.E.M. derived from 5-16 cells.

Fig. 4. Effects of high frequency stimulation of the firing of cholinergic interneurons

A, Cell-attached recording of spontaneous action potential firing in a cholinergic interneuron of a wild type mouse (WT, P23) under control condition (Control), just after 10 Hz stimulation for 10 s (10 Hz), and after 10 Hz stimulation for 10 s in the presence of sulpiride

(10 μ M, 10 Hz in sulpiride). Firing was inhibited by 10 Hz stimulation and the inhibition was antagonized by sulpiride. **B**, Cell-attached recording of in a cholinergic interneuron of a D2R KO mouse (D2R KO, P23) under control condition (Control) and just after 10 Hz stimulation for 10 s (10 Hz). The stimulation had no effect in the D2R KO mouse. **C**, Summarized histograms showing the 5 and 10 Hz (5 and 10 below the hatched histograms, respectively) stimulation-induced inhibition of the action potential firing, antagonism by sulpiride, and lack of the effect in D2R KO mice. *, $P < 0.05$ with paired *t*-test compared with the firing rate in control condition (white histogram).

Fig. 5. Effects of ω -CgTX and ω -Aga-TK on the IPSCs evoked in striatal cholinergic interneurons

A, Time course of inhibitory effect of ω -CgTX (3 μ M) and subsequent application of ω -Aga-TK (200 nM) on the IPSCs in a wild type (WT, a) and a D2R KO mouse (D2KO, b). Toxins were bath applied during the indicated periods. Each point in the time course plot represents the amplitude of an individual IPSC evoked at 0.2 Hz (every 5s) in the presence of CNQX (5 μ M), D-AP5 (25 μ M) and strychnine (0.5 μ M) with CsCl-based internal solution. The holding potential was -60 mV. Superimposed traces on the right are averages of 20 consecutive IPSCs during the indicated periods. **B**, Time course of the effect of ω -Aga-TK and subsequent application of ω -CgTX on the IPSCs in a WT (a) and a D2R KO mouse (b) **C**,

Summarized histograms showing the inhibitory effect of ω -CgTX on WT and D2R KO mice. Numbers of cells tested are indicated in parentheses. The effect of ω -CgTX was significantly smaller in D2R KO mice than in wild type mice (* $P < 0.05$). **D**, Summarized histograms showing the inhibitory effect of ω -Aga-TK on WT and D2R KO mice. Effect of ω -Aga-TK was significantly ($P < 0.05$) larger in D2R KO mice than in WT mice (*). **E**, Developmental changes in ω -CgTX-induced effect in WT and D2R KO mice. The value of WT at P20-23 (young) is identical in Fig. 5C. The ω -CgTX-induced inhibition of IPSCs was significantly ($P < 0.05$, *) smaller at P35-44 (adult) than young group. On the other hand, the ω -CgTX-induced inhibition was not significantly ($P > 0.05$) different between young and adult D2R KO mice.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5