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Increased desensitization of dopamine D_2 receptor–mediated response in the ventral tegmental area in the absence of adenosine A_{2A} receptors

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Abstract

G-protein coupled receptors interact to provide additional regulatory mechanisms for neurotransmitter signaling. Adenosine A_{2A} receptors are expressed at a high density in striatal neurons, where they closely interact with dopamine D_2 receptors and modulate effects of dopamine and responses to psychostimulants. A_{2A} receptors are expressed at much lower densities in other forebrain neurons but play a more prominent yet opposing role to striatal receptors in response to psychostimulants in mice. It is, therefore, possible that A_{2A} receptors expressed at low levels elsewhere in the brain may also regulate neurotransmitter systems and modulate neuronal functions. Dopamine D_2 receptors play an important role in autoinhibition of neuronal firing in dopamine neurons of the ventral tegmental area (VTA) and dopamine release in other brain areas. Here, we examined the effect of A_{2A} receptor deletion on D_2 receptor-mediated inhibition of neuronal firing in dopamine neurons in the VTA. Spontaneous activity of dopamine neurons was recorded in midbrain slices, and concentration-dependent effects of the dopamine D_2 receptor agonist, quinpirole, was compared between wild-type and A_{2A} knockout mice. The potency of quinpirole applied in single concentrations and the expression of D_2 receptors were not altered in the VTA of the knockout mice. However, quinpirole applied in stepwise escalating concentrations caused significantly reduced maximal inhibition in A_{2A} knockout mice, indicating an enhanced agonist-induced desensitization of D_2 receptors in the absence of A_{2A} receptors. The A_{2A} receptor agonist, CGS21680, did not exert any effect on dopamine neuron firing or response to quinpirole, revealing a novel non-pharmacological interaction between adenosine A_{2A} receptors and dopaminergic neurotransmission in midbrain dopamine neurons. Altered D_2 receptor desensitisation may result in changes in dopamine neuron firing rate and pattern and dopamine

release in other brain areas in response to persistent dopamine release and administration of psychostimulants.

Keywords. adenosine A_{2A} receptor, dopamine D_2 receptor, dopamine neuron, D_2 receptor desensitization, A_{2A} receptor knockout mice, ventral tegmental area

Abbreviations.

CGS21680, 4-[2-[[6-amino-9-[(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride

SCH23390, (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride

VTA, ventral tegmental area

Introduction

Increasing numbers of studies show that G-protein coupled receptor (GPCR) signaling can occur through the formation of oligo-dimers or multimers in neurons (Dalrymple *et al.*, 2008; Ferre *et al.*, 2008). Adenosine A_{2A} receptors are $G_{s/olf}$-coupled receptors for the endogenous neuromodulator adenosine (Fredholm *et al.*, 2011). The expression of A_{2A} receptors in the brain is at the highest density in the soma of the GABAergic medium spiny neurons in the striatum, with significantly lower levels of expression elsewhere in the brain (Rosin *et al.*, 1998; Svenningsson *et al.*, 1999; Rosin *et al.*, 2003). In the striatum, A_{2A} receptors co-localize with dopamine D_2 receptors (Ferre *et al.*, 2008). Via different G-protein coupling and second messenger systems, the activation of the adenosine A_{2A} receptors increases cAMP levels and cellular excitability, while the activation of the dopamine D_2 receptors decreases cAMP levels and cellular excitability. Antagonistic interactions between the two receptor systems are also manifested by direct receptor-receptor cross-talk in the form of heterodimers, where stimulation of the adenosine A_{2A} receptors causes a reduction in the affinity of dopamine D_2 receptor agonists and vice versa (Ferre *et al.*, 1991; Kull *et al.*, 1999; Azdad *et al.*, 2009). The functional interactions between adenosine A_{2A} receptors and dopamine D_2 receptors are thought to underlie the involvement of adenosine A_{2A} receptors in the control of several behavioral functions (Sebastiao & Ribeiro, 1996; Fredholm *et al.*, 2005). Most notably, A_{2A} receptor agonists inhibit the motor and rewarding effects of psychostimulants, similar to effects produced by dopamine D_2 receptor antagonists; whereas A_{2A} receptor antagonists potentiate effects of psychostimulants, in agreement with the activation of dopamine D_2 receptors (Rimondini *et al.*, 1997; Shimazoe *et al.*, 2000; Knapp *et al.*, 2001; Filip *et al.*, 2006).

In adenosine A_{2A} receptor knockout mice (Ledent *et al.*, 1997), however, both the motor and reward effects of psychostimulants were reduced (Chen *et al.*, 2000; Fredholm *et al.*, 2005; Soria *et al.*, 2005; Castane *et al.*, 2006; Soria *et al.*, 2006; Shen *et al.*, 2008), contrary to the enhancing effects of A_{2A} receptor antagonists in wild-type mice. The discord between effects of A_{2A} receptor ligands and genetic silencing raises potential issues regarding the long-term use of A_{2A} receptor antagonists for the treatment of Parkinson's disease (Schwarzschild *et al.*, 2006) and A_{2A} agonists for schizophrenia (Ferre, 1997). Compensatory alterations in neural circuits are one of the possible causes for the different effects of psychostimulants in the A_{2A} receptor knockout mice. However, extrastriatal A_{2A} receptors, for example those located on glutamatergic inputs to the striatum from the cerebral cortex (Schiffmann *et al.*, 2007), albeit at low expression density, were found to mediate a prominent excitatory effects of psychostimulants, opposite to the effects of postsynaptic striatal A_{2A} receptors on striatopallidal neurons (Shen *et al.*, 2008). The use of striatum and forebrain-specific A_{2A} receptor knockout mice has revealed that extrastriatal A_{2A} receptors in the forebrain predominently mediate the excitatory effects of psychostimulants, while striatal A_{2A} receptors mediate inhibitory effects of psychostimulants (Shen *et al.*, 2008). These findings may suggest that A_{2A} receptors that are expressed outside the striatum, albeit at low densities, could play significant roles in neuronal functions.

The ventral tegmental area (VTA) is a midbrain region closely involved in dopaminergic neurotransmission, as dopaminergic neurons in the VTA form ascending mesocorticolimbic projections to forebrain regions. In addition, the VTA is suggested to be the site important for the development of sensitization to repeated

exposures to drugs of abuse, via effects of dopamine on dopamine receptors (Wise, 1996) and neuroplastic changes at glutamatergic synapses (Bonci & Malenka, 1999). Dopamine D_1- and D_2-like receptors (referred to as D_1 and D_2 receptors thereafter) are highly expressed in the VTA (Bouthenet *et al.*, 1987; Wamsley *et al.*, 1989; Chen *et al.*, 1991; Adell & Artigas, 2004), where activation of D_2 receptors inhibits the firing activity of dopamine neurons, carrying out an important autoinhibitory function (Bunney *et al.*, 1973; White & Wang, 1984; Sibley *et al.*, 1993; Mercuri *et al.*, 1997; Adell & Artigas, 2004).

We examined the effects of A_{2A} receptor deletion on D_2 receptor-mediated autoinhibition of VTA dopamine neuron firing activity in brain slices. Concentration-dependent effects of the D_2 receptor-selective agonist, quinpirole, were investigated in wild-type and the global adenosine A_{2A} receptor knockout mice. Expression of dopamine receptors in the VTA of the A_{2A} knockout mice and effects of the A_{2A} receptor agonist CGS21680 on neuronal firing and responses to quinpirole were also assessed. We found that the maximal effect of quinpirole was reduced in the A_{2A} receptor knockout mice when treated with rising concentrations of quinpirole, but not single concentrations, which may indicate enhanced agonist-induced desensitization of D_2 receptors in the absence of A_{2A} receptors. A novel role of A_{2A} receptors in modulating dopaminergic neurotransmission is revealed.

Experimental Procedures

All animal breeding and experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

VTA slice preparation and electrophysiological recordings

The adenosine A_{2A} receptor knockout mice on the out-bred CD1 background were originally created by Ledent *et al.* (1997) and heterozygous breeding colonies are maintained at the University of Surrey. Midbrain slices were prepared from wildtype and adenosine A_{2A} receptor knockout mice aged 3-4 weeks. The mice were killed by cervical dislocation and the whole brains were immediately dissected and immersed in ice cold sucrose-containing buffer and saturated with 95% O_2/5% CO_2. The sucrose-containing buffer contained: 200 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25 mM $NaHCO_3$, 10 mM $MgSO_4$, 2.5 mM KCl, 1.2 mM NaH_2PO_4 and 0.5 mM $CaCl_2$. The midbrain block was dissected and mounted with cyanoacrylic glue to a glass cutting stage of a vibratome (Series 1000 Vibratome, St. Louis, Missouri, USA). Single coronal sections (300 μm) containing the VTA were harvested (3-4 per animal) and placed in an incubation chamber for at least 1 hour at 27-28˚C in oxygenated aCSF, which contained NaCl 123 mM, $NaHCO_3$ 22 mM, NaH_2PO_4 1.25 mM, KCl 3.75 mM, D-Glucose 10 mM, $CaCl_2$ 2.5 mM and $MgSO_4$ 1.2 mM.

Electrophysiological recordings were performed as previously detailed for rat VTA slice preparations (Chen *et al.*, 2005; de Filippi *et al.*, 2010). A single slice was placed in the recording chamber and continuously perfused with oxygenated aCSF at a flow rate of 2 ml/min and at 32 ± 0.5°C. The VTA area was identified as a grey area medial

to the substantia nigra and the medial lemniscus. Borosilicate glass microelectrodes with a tip of approximately 1 μm, pulled with a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, California, USA), were filled with aCSF to give an impedance of 3-6 MΩ and used for recording from single neurons. Extracellular recordings of the spontaneous action potentials were amplified (AxonPatch 1D, Molecular Devices Corp., Silicon Valley, CA, USA) initially by 10 times in the I = 0 mode with a low-cut off frequency at 5 kHz and then by 100 times in the AC mode (Neurolog systems, Digitimer, Welwyn Garden City, UK) without any further filtering. Signals were digitised using CED1401 Micro Mark 2 (Cambridge Electronic Design Ltd, Cambridge, UK), and captured with Spike 2 Software Version 5.2 (Cambridge Electronic Design Ltd, Cambridge, UK).

Two types of spontaneously firing neurons in the VTA were recorded and identified. Dopamine neurons were classically defined by their pacemaker-like spontaneous firing, with frequencies at 0.5 to 4 Hz and an action potential waveform of 2.5 – 3 ms in duration with a large negative phase. The firing frequency was significantly suppressed by 50 μM dopamine (-74.2 ± 4.8%, n = 29) or by the D_2 agonist, quinpirole (Grace & Onn, 1989; Johnson & North, 1992). The other type of neurons displayed higher spontaneous firing frequencies (4 – 15 Hz) with action potential waveforms of a shorter duration (~ 2ms). Dopamine did not inhibit the firing of these classically defined non-dopaminergic or GABAergic neurons (Grace & Onn, 1989; Johnson & North, 1992). A recent study confirmed that almost all electrophysiologically-classified dopaminergic neurons are tyrosine hydroxylase-positive (Brown *et al.*, 2009), despite discrepancies found in some other studies (Margolis *et al.*, 2006; Margolis *et al.*, 2010). “Dopamine neurones” referred to in this

study are therefore those that conform to electrophysiological and pharmacological criteria, but not necessarily to the content of dopamine.

Autoradiographic binding

The brains of wild-type and adenosine A_{2A} receptor knockout mice were removed, snap frozen in isopentane (-35˚C) and stored at -80˚C until sectioning. Adjacent 20 μm coronal sections were cut at an interval of 300 μm for the determination of total and non-specific binding (NSB). Quantitative autoradiography was performed as detailed previously for dopamine D_1 and D_2 receptor binding (Lena *et al.*, 2004) and dopamine transporters (Javitch *et al.*, 1985; Bailey *et al.*, 2007) using [^{3}H]SCH23390, [^{3}H]raclopride and [^{3}H]mazindol, respectively. The ligand concentration (4 nM) was 3-4 times K_d. Non-specific binding (NSB) was defined in the presence of 10 μM cis-flupenthixol for D_1, sulpiride for D_2 and non-tritiated mazindol for dopamine tranporter. Quantitative analysis of brain receptors was performed as detailed previously (Kitchen *et al.*, 1997; Bailey *et al.*, 2004b; Lena *et al.*, 2004) using an MCID image analyser (ImageResearch, Canada).

Statistical Analysis

The mean and standard error of the mean were presented. Comparison of quantitative measures was carried out, where appropriate, using Student's *t*-test or two-way ANOVA followed by a Newman Keuls post-hoc analysis. Only one slice was used from each mouse for each set of experiment, so that the n numbers given represent both the number of neurons and the number of mice.

Materials

Quinpirole and CGS21680 were purchased from Tocris Bioscience (Bristol, Somerset, UK), and sulpiride and NECA were supplied by Sigma-Aldrich Company Ltd (Dorset, UK). [^{3}H]raclopride (60.1 Ci/mmol), [^{3}H]SCH23390 (85.0 Ci/mmol) and [^{3}H]mazindol (17.8 Ci/mmol) were purchased from Perkin Elmer (Buckinghamshire, UK). The [^{3}H] microscales were purchased from Amersham International (Buckinghamshire, UK). The autoradiographic film, developer and fixer were supplied by Sigma-Aldrich Company Ltd (Dorset, UK).

Results

Potency of quinpirole on dopamine neuron firing is similar between wild-type and A_{2A} receptor knockout mice

To examine the function of dopamine D_2 receptors in adenosine A_{2A} receptor knockout mice, concentration-dependent effects of the D_2 receptor agonist, quinpirole, were examined on dopamine neuron firing in midbrain slices. Bath application (15 min) of single concentrations of quinpirole ranged between 1 nM and 1 μM inhibited dopamine neuron firing rate concentration-dependently, in agreement with previous reports (Bowery *et al.*, 1994; Mercuri *et al.*, 1997; Centonze *et al.*, 2002). Complete inhibition was achieved in both wild-type (Fig. 1A, n = 4-7 per data point) and adenosine A_{2A} receptor knockout mice (Fig. 1B, n = 3-5 per data point), and the EC_{50} values were not different between the wild-type (11.7 nM or $LogEC_{50}$ = -1.93 ± 0.15 μM) and knockout mice (14.5 nM or $LogEC_{50}$ = -1.84 ± 0.04 μM, $P > 0.05$, two-way ANOVA), showing the similar potencies of quinpirole on D_2 receptors in dopamine neurons of A_{2A} receptor knockout mice.

In addition, the baseline firing frequency and the pacemaker firing pattern of dopamine neurons were also similar ($P > 0.05$; Student's t-test) between wild-type (1.8 ± 0.2Hz, n = 22) and adenosine A_{2A} receptor knockout mice (1.8 ± 0.3Hz, n = 14). Action potential waveforms were also unchanged, indicating unaltered basic electrophysiological characteristics in dopamine neurons (data not shown). The D_2 receptor antagonist sulpiride (10 μM) blocked the effects of quinpirole in both genotypes without altering baseline firing rate (data not shown), confirming that the effect of quinpirole was mediated by D_2 receptors (Bernardini *et al.*, 1991; Bowery *et al.*, 1994; Mercuri *et al.*, 1997; Centonze *et al.*, 2002), and that the baseline firing rate

of dopamine neurons *in vitro* was not affected by D_2 receptors (Grace & Onn, 1989; Johnson & North, 1992).

Reduced cumulative effect of quinpirole on dopamine neuron firing in A_{2A} receptor knockout mice

Although the effects of D_2 receptor activation in response to single concentrations of quinpirole were not different between wild-type and A_{2A} receptor knockout mice, rising concentrations of dopamine (Nimitvilai & Brodie, 2010), prolonged synchronous stimulation of dopamine neurons (Beckstead & Williams, 2007) and administration of cocaine (Di Chiara, 1995; Ford *et al.*, 2010) may cause receptor desensitisation (Beckstead & Williams, 2007). To examine the effect of A_{2A} receptor deletion on D_2 receptor desensitisation, quinpirole was applied cumulatively in rapidly escalating concentrations (0.01 – 30 μM, 5 min at each concentration). The EC_{50} value for quinpirole applied in this way was approximately 4 times higher than that at single concentrations (44.9 vs. 11.7 nM in wild-type mice), showing reduced sensitivity of D_2 receptors probably due to agonist-induced receptor desensitisation. The EC_{50} values were again not different between wild-type and knockout mice (44.9 nM or $LogEC_{50}$ = -1.34 ± 0.05 μM vs. 44.9 nM or $LogEC_{50}$ = -1.35 ± 0.13, Fig. 2). However, the maximal effects of quinpirole were different. In a majority of the wild-type mice (n = 10/11), quinpirole induced complete inhibition of firing rate, similar to single concentration experiments; whereas in more than half of the A_{2A} receptor knockout mice, the firing rates were only partially blocked by the D_2 agonist (n = 4/7, Fig. 2B). As a result, the mean maximal response in the A_{2A} receptor knockout mice (81.2% ± 4.8, n = 7, $P < 0.05$, Students' t-test) was significantly reduced compared to the wild-type mice (96.5% ± 2.3, n = 11), showing a significantly greater reduction of

D_2 receptor function in the absence of A_{2A} receptors. The effects of quinpirole in both genotypes were prevented by sulpiride (10 μM, Fig. 2A), confirming D_2 receptor-mediated effects. The absence of A_{2A} receptors, therefore, increased agonist-induced D_2 receptor desensitisation in dopamine neurons.

No change in the expression of dopamine D_1 and D_2 receptors and dopamine transporters in the A_{2A} receptor knockout mice

Although the potency of quinpirole was similar in both genotypes, it is unknown whether the expression of D_2 receptors was changed in dopamine neurons of the A_{2A} receptor knockout mice. Previous studies from our and other groups identified no change in D_2 receptor densities in the striatal quadrant of the adenosine A_{2A} receptor knockout brains despite significant increases in dopamine D_1, D_2 and DAT mRNAs (Chen *et al.*, 2001; Dassesse *et al.*, 2001; Bailey *et al.*, 2004a; Short *et al.*, 2006), but their expressions in the VTA were not examined. Here, we examined the expression of dopamine receptors in the midbrain regions by autoradiography.

D_2 receptor density was measured by [^{3}H]raclopride binding in the VTA and substantia nigra (Fig. 3 lower panels), nucleus accumbens and caudate putamen (Fig. 3 upper panels), as well as olfactory tubercle. Overall, the results show a lack of statistically significant differences in any single brain regions, including the VTA, in the knockout brain, despite a small but significant genotype effect overall (Table 1, $P < 0.05$, Two-way ANOVA). In addition, D_1 receptor and dopamine transporter binding in any of the brain regions investigated were not significantly changed (Table 1), indicating their unaltered expression levels. The unaltered D_2 receptor expression in the VTA in A_{2A} receptor knockout mice is in agreement with the similar potencies

of quinpirole found on firing rate inhibition, showing unchanged D_2 receptors function in the VTA.

The lack of functional A_{2A} receptors in dopamine neurons of the VTA

In order to understand how the deletion of A_{2A} receptors affects D_2 receptor function, we examined for any functional role of adenosine A_{2A} receptors on dopamine neuron firing and on effects of quinpirole. The A_{2A} receptor-selective agonist, CGS21680, applied for 10 min in the aCSF at concentrations of 0.001, 0.01, 0.1, 1, 10 and 30 μM, caused no significant effects on baseline firing frequency or pattern in the wild-type mice ($P > 0.05$, Student's paired t-test, $n = 2$-4 for each concentration, data not shown), showing a lack of direct effect of A_{2A} receptor activation on dopamine neurons.

Effects of CGS21680 (1 μM, 10 min pre-treatment) on quinpirole-induced firing rate inhibition were also examined in the wild-type mice. No significant difference was seen on the effects of 30 nM quinpirole, a submaximal concentration, with 64.8 ± 13.2% inhibition before and 64.9 ± 19.3% after the treatment with CGS21680 ($n = 5$, $P > 0.05$, paired t-test). Direct pharmacological interactions between A_{2A} and D_2 receptors were, therefore, not detected on dopamine neurons.

Discussion

The results show a decreased use-dependent function of dopamine D_2 receptors in midbrain dopamine neurons in the absence of adenosine A_{2A} receptors. The dopamine D_2 receptor agonist quinpirole exhibited similar potencies on the inhibition of dopamine neuron firing rate between wild-type and adenosine A_{2A} knockout mice when applied at single concentrations, with D_2 receptor binding not significantly altered in the VTA. However, the maximum effect of quinpirole, when applied cumulatively at increasing concentrations, was reduced to a greater extent in the adenosine A_{2A} receptor knockout mice, indicating increased agonist-induced D_2 receptor desensitisation in the absence of A_{2A} receptors. As D_2 receptors serve an important autoinhibitory role in the regulation of dopamine neuron firing, reduction of D_2 receptor function could affect firing rate and pattern of dopamine neurons in a use-dependent manner and alter action potential-dependent dopamine release in other brain areas *in vivo*.

Activation of D_2 receptors stimulates G-proteins (Gi/o) leading to opening of G-protein–coupled inward rectifying potassium (GIRK) channels, which hyperpolarises the membrane potential and reduces the firing rate of dopamine neurons (Grace & Onn, 1989). The efficacy of agonist-induced reduction of dopamine neuron firing rate can, therefore, be modulated by several mechanisms. One way is through reduction in receptor availability, by reducing receptor expression or accelerating receptor internalisation, which involves endocytosis of agonist-bound receptors via phosphorylation by G-protein receptor kinases and binding with β-arrestin (Hanyaloglu & von Zastrow, 2008). Other mechanisms may involve changes in G-protein availability, G-protein coupling to GIRK channels, and the availability and

activity of GIRK channels (Werner *et al.*, 1996). As the effects of quinpirole tested in single concentrations were not different between wild-type and A_{2A} receptors knockout mice (Fig. 1), and there was no change in the total number of receptors under normal conditions (Table 1), D_2 receptor availability and coupling to effectors were probably unaltered. The reduced maximal effect of accumulative quinpirole in A_{2A} receptors knockout mice (Fig. 2) was, therefore, most likely caused by increased receptor desensitisation with the hallmark of use-dependency (Hanyaloglu & von Zastrow, 2008).

D_2 receptor desensitisation is not normally apparent when agonists are used to inhibit dopamine neuron firing *in vitro* (Fig. 1) (Bowery *et al.*, 1994; Mercuri *et al.*, 1997; Centonze *et al.*, 2002) or *in vivo* (Bunney *et al.*, 1973; White & Wang, 1984; Kalivas *et al.*, 1993; Sibley *et al.*, 1993; Adell & Artigas, 2004), in agreement with their role of providing essential feedback autoinhibition on dopamine neurons. However, desensitisation can be induced with high concentrations of agonists (Beckstead & Williams, 2007), in particular with accumulative dosing (Fig. 2) (Nimitvilai & Brodie, 2010), showing agonist and concentration-dependency of receptor internalisation. Furthermore, desensitization of D_2 receptors in the VTA was enhanced by concurrent activation of D_1/D_5 receptors (Nimitvilai & Brodie, 2010), indicating modulation by other receptors. Here, we show that deletion of adenosine A_{2A} receptors enhances D_2 receptor desensitization induced by accumulative dosing of quinpirole in the VTA, indicating interaction between the two receptors on D_2 receptor trafficking (Hanyaloglu & von Zastrow, 2008).

A_{2A} receptors are known to have high affinities for D_2 receptors (Ferre *et al.*, 2008) and their heterodimers also have the potential to internalise differentially from each homomers and, thereby, desensitise differentially (Hillion *et al.*, 2002; Torvinen *et al.*, 2005; Vidi *et al.*, 2008). We also show here that the A_{2A} receptor agonist, CGS21680, did not cause any pharmacological effect on the firing rate or pattern of dopamine neurons, confirming the lack of pharmacologically accessible A_{2A} receptors on dopamine neurons, which is in agreement with a low level of A_{2A} receptor expression in the VTA (Rosin *et al.*, 1998; Rosin *et al.*, 2003). CGS21680 also failed to acutely alter D_2 receptor function in the VTA, indicating a lack of direct pharmacological interaction between D_2 and A_{2A} receptors in the VTA, like those in the striatum (Azdad *et al.*, 2009).

One possible explanation for the results is that A_{2A} receptors are not expressed as functional receptors in dopamine neurons but participate in D_2 receptor trafficking. Although GPCR heterodimerisation tends to cause cross-conformational switch resulting in changes in receptor affinity for pharmacological ligands (Dalrymple *et al.*, 2008; Ferre *et al.*, 2008), a recent study showed that the association of $GABA_{B2}$ with muscarinic M_2 receptors selectively prevented M_2 receptor internalization in PC12 cells without altering the dose-response of M_2 receptor agonists (Boyer *et al.*, 2009). Based on this model, it is therefore possible to speculate that A_{2A} receptors may interface between D_2 receptors and G-protein-coupled receptor-associated sorting proteins (Bartlett *et al.*, 2005) to facilitate membrane insertion of D_2 receptors. Indeed in the A_{2A} receptor knockout mice, D_2 receptor expression at the mRNA level were found increased, but not the number of functional receptors (Chen *et al.*, 2001; Dassesse *et al.*, 2001; Bailey *et al.*, 2004a; Short *et al.*, 2006). This scenario could be

explained by the presence of a larger proportion of internalised D_2 receptors in the absence of A_{2A} receptors.

On the other hand, interaction between forebrain A_{2A} receptors and VTA neuronal activity may also occur via neural network connections. Dopamine neurons in the VTA project to nucleus accumbens and the prefrontal cortex and these target areas also innervate the VTA via GABAergic and glutamatergic feedback loops, respectively (Kalivas *et al.*, 1993; Pierce & Kumaresan, 2006). High density expression of A_{2A} receptors is found in the nucleus accumbens. Although A_{2A} receptors are predominantly expressed in association with D_2 receptors in GABAergic enkephalinergic neurons, not the dynorphinergic neurons that form the GABAergic accumbens-mesencephic projections (Fenu *et al.*, 1997; Lu *et al.*, 1997; Svenningsson *et al.*, 1999), deletion of A_{2A} receptors in the nucleus accumbens may still indirectly affect the activities of dopamine neurons in the VTA. On glutamatergic terminals, A_{2A} receptors were shown to modulate glutamate release in the striatum (Schiffmann *et al.*, 2007) and mediate a prominent excitatory effect of psychostimulants (Shen *et al.*, 2008). It is unknown whether A_{2A} receptors are expressed on glutamatergic terminals in the VTA, but a recent study indicated a role for forebrain A_{2A} receptors in the VTA, as deletion of forebrain A_{2A} receptors promoted survival of midbrain dopamine neurons in response to MPTP toxicity in mice (Carta *et al.*, 2009). Assuming the existence of presynaptic A_{2A} receptors on glutamatergic terminals in the VTA, their presynaptic modulation of glutamate release may alter the strength of glutamatergic synaptic transmission (Mansvelder & McGehee, 2000) and the activity state of dopamine neurons, which may subsequently regulate D_2 receptor trafficking.

Whatever the mechanism underlying interaction between D_2 and A_{2A} receptors in dopamine neurons, reduced D_2 receptor-mediated autoregulation of dopamine neuron activity in the VTA may contribute significantly to the dopaminergic state in A_{2A} receptor knockout mice (Chen *et al.*, 2000; Fredholm *et al.*, 2005; Soria *et al.*, 2005; Castane *et al.*, 2006; Soria *et al.*, 2006; Shen *et al.*, 2008). D_2 receptors are not activated during rhythmic single spike firing *in vitro* (Grace & Onn, 1989), but significantly contribute to burst firing of action potentials *in vivo* together with the intrinsic ion channel properties of dopamine neurons (Grace & Bunney, 1984) and excitatory inputs from other brain areas, with the activation of glutamatergic N-methyl-D-aspartate receptors being most prominent (Overton & Clark, 1997). Synchronized high-frequency stimulation of dopamine neurons can activate D_2 receptors by increasing somatodendritic release of dopamine (Bunney *et al.*, 1973; White & Wang, 1984; Sibley *et al.*, 1993; Mercuri *et al.*, 1997; Adell & Artigas, 2004), with the resultant inhibitory current inhibits the firing of dopamine neurons generating a "pause" of firing (Beckstead *et al.*, 2004). The presence of D_2 receptor antagonist haloperidol, therefore, impaired burst by inducing depolarization block of firing (Grace & Bunney, 1986). Consequently, the release of dopamine in the striatum was reduced (Moore *et al.*, 1998), further demonstrating the role of D_2 receptors in homeostatic regulation of dopamine neuron activity and the release of dopamine in the nucleus accumbens and prefrontal cortex that mediate many of the behavioural consequences of dopamine release, particularly in response to reward (Schultz, 2002).

Increased D_2 receptor desensitisation also reduces autoinhibition in dopamine neurons, which could, subsequently affect the generation of burst activity. Consequently, dopamine release in other brain regions may be reduced, causing a hypodopaminergic

state reported previously in A_{2A} receptor knockout mice (Chen *et al.*, 2000; Fredholm *et al.*, 2005; Soria *et al.*, 2005; Castane *et al.*, 2006; Soria *et al.*, 2006; Shen *et al.*, 2008).

Conclusions

Increased dopamine D_2 receptor desensitisation is revealed in dopamine neurons of the VTA in A_{2A} receptor knockout mice, revealing a novel interaction between dopamine D_2 and adenosine A_{2A} receptors outside the striatum. The reduced D_2 receptor function may contribute to the hypodopaminergic state reported in A_{2A} receptor knockout mice due to reduced D_2 receptor-mediated regulation of dopamine neuron activity and subsequent dopamine release. Understanding A_{2A} and D_2 receptor interaction in midbrain dopamine neurons may shed new light on the roles of A_{2A} receptors in dopamine related neurological and psychiatric disorders, such as drug addiction, schizophrenia and Parkinson's disease, and on treatment strategies targeting the A_{2A} receptors .

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Figure legend

Figure 1. Quinpirole, a dopamine D_2 receptor agonist, causes concentration-dependent inhibition of the spontaneous firing rate of putative dopamine neurons in the VTA. Representative traces illustrate the similar effects of 0.1 μM quinpirole on the firing rate of dopamine neurons recorded in brain slices prepared from wild-type (A. WT) and A_{2A} receptor knockout mice (B. KO). Firing frequency (Hz) was calculated from 10s bins. C. The concentration-dependent inhibition of firing rate induced at different concentrations of quinpirole is compared between the WT and KO mice. There is no statistical significance between genotypes ($P > 0.05$, two-way ANOVA).

Figure 2. Reduced inhibition of firing by quinpriole applied in cumulative concentrations in the A_{2A} receptor knockout mice. A. Quinpirole applied in escalating concentrations in a cumulative manner induced complete inhibition of the firing rate of dopamine neurons in the wild-type (WT), but the effect was reduced in the A_{2A} receptor knockout (KO) mice. The application of sulpiride (10 μM) blocked the effect of quinpirole. Firing frequency was calculated from 10s bins. B. A comparison of the accumulative concentration-dependent effects of quinpirole in WT ($n = 11$) and KO ($n = 7$) mice shows significantly reduced maximal effect in the KO mice ($P < 0.05$, two-way repeated measures ANOVA).

Figure 3. Representative autoradiograms of dopamine D_2 receptor ligand [^{3}H]raclopride in brain sections of wild-type (A. WT) and A_{2A} receptor knockout mice (B. A_{2A} KO) show similar binding density. Total binding was obtained using 4 nM [^{3}H]raclopride at 3-4 times K_d. Non-specific binding (NSB) was defined in the

presence of 10 μM sulpiride. The colour bar shows the calibrated density of radioligand binding (fmol/mg) in black and white film images. Selective binding is obtained (see Table 1) by subtracting NSB from the total binding. Upper panels show coronal brain sections at the level of striatum and lower panels contain midbrain structures showing labelling in the ventral tegmental area and the substantia nigra. Sections from WT and KO mice were processed in parallel.

Fig. 1

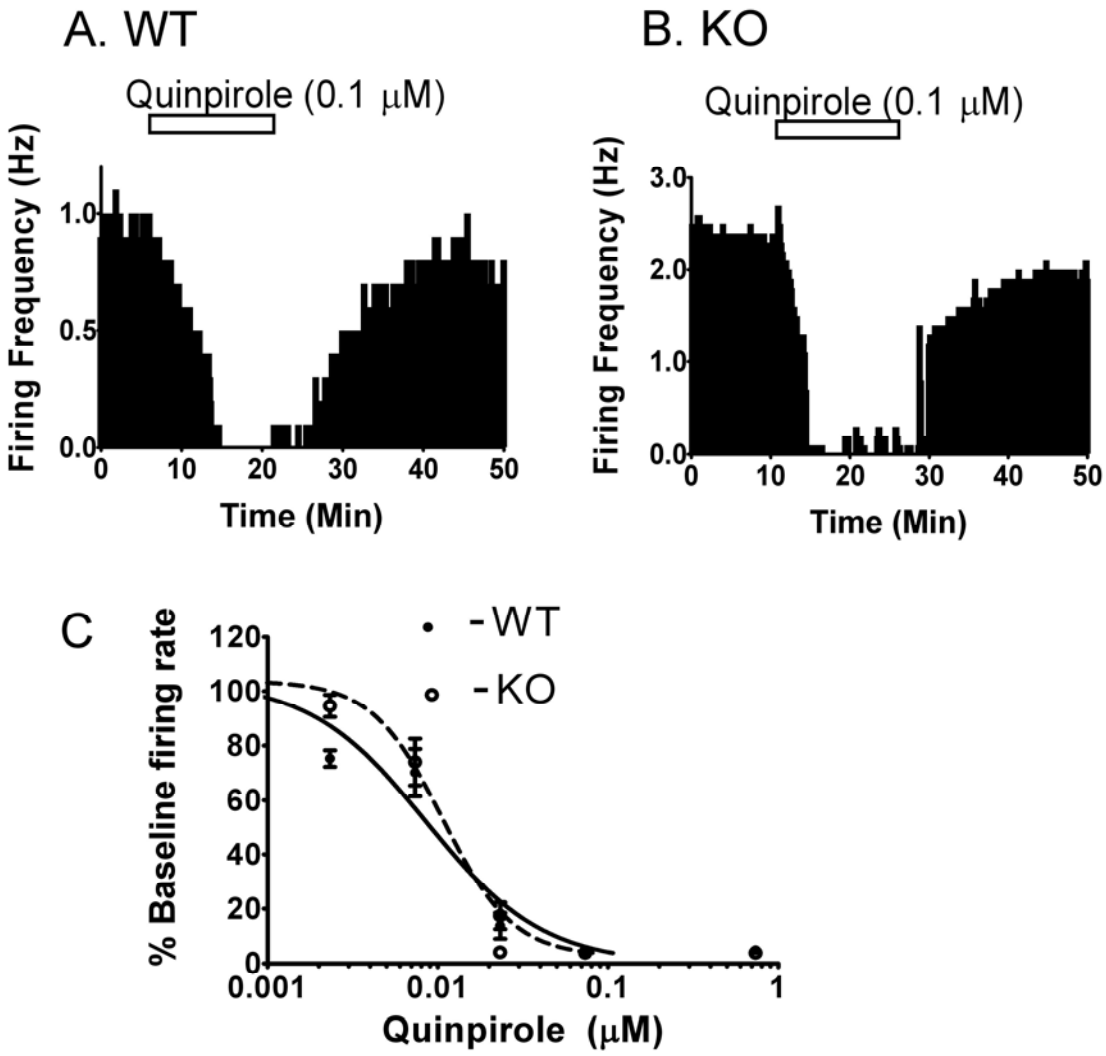
A. WT
Quinpirole (0.1 μM)
Firing Frequency (Hz)
1.0
0.5
0.0
0
10
20
30
40
50
Time (Min)
B. KO
Quinpirole (0.1 μM)
Firing Frequency (Hz)
3.0
2.0
1.0
0.0
0
10
20
30
40
50
Time (Min)
C
-WT
-KO
% Baseline firing rate
120
100
80
60
40
20
0
0.001
0.01
0.1
1
Quinpirole (μM)

Fig. 2

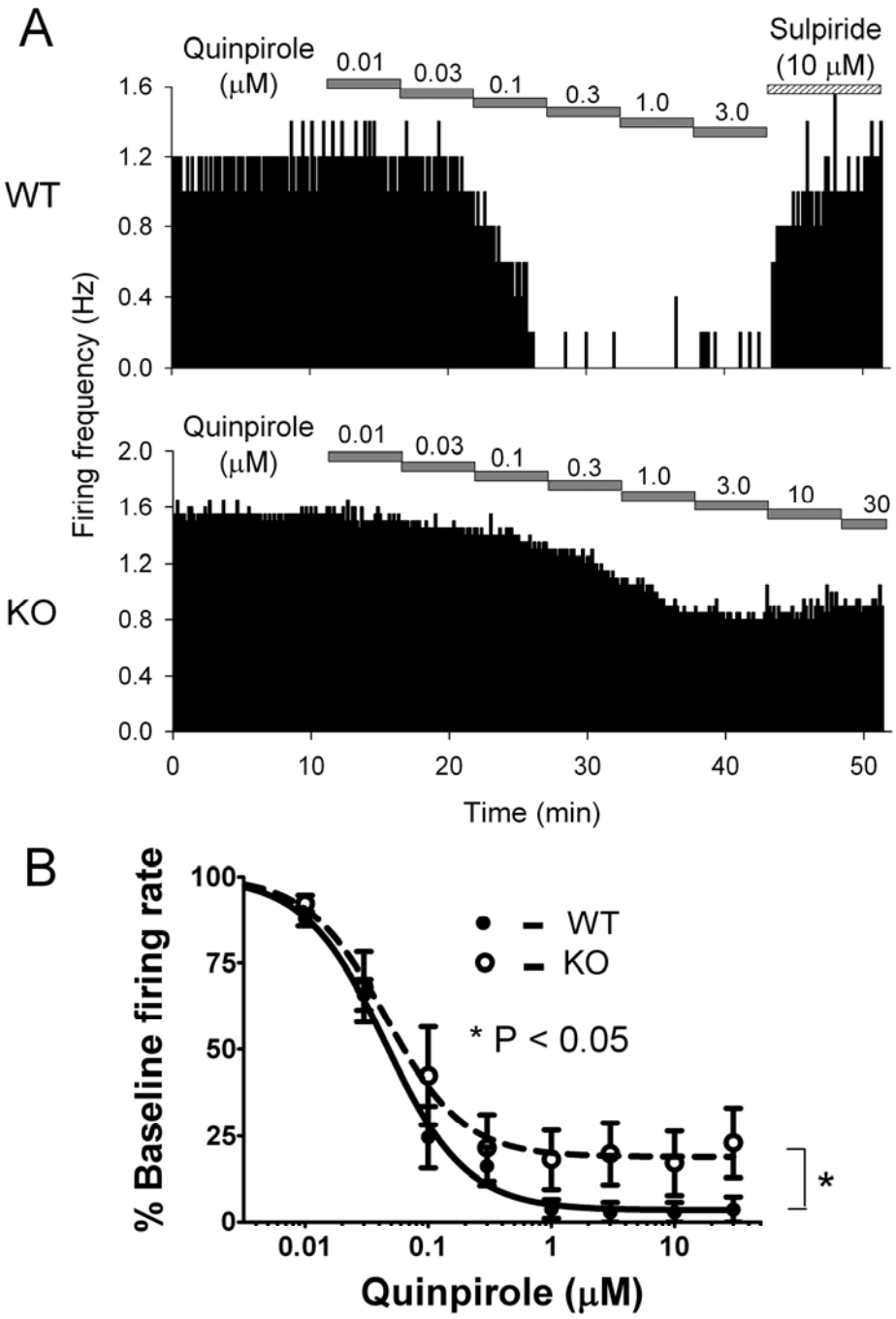
A
Quinpirole (μM)
0.01
0.03
0.1
0.3
1.0
3.0
Sulpiride (10 μM)
WT
KO
10
30
Firing frequency (Hz)
Time (min)
B
% Baseline firing rate
WT
KO
* P < 0.05
*
Quinpirole (μM)

Fig. 3

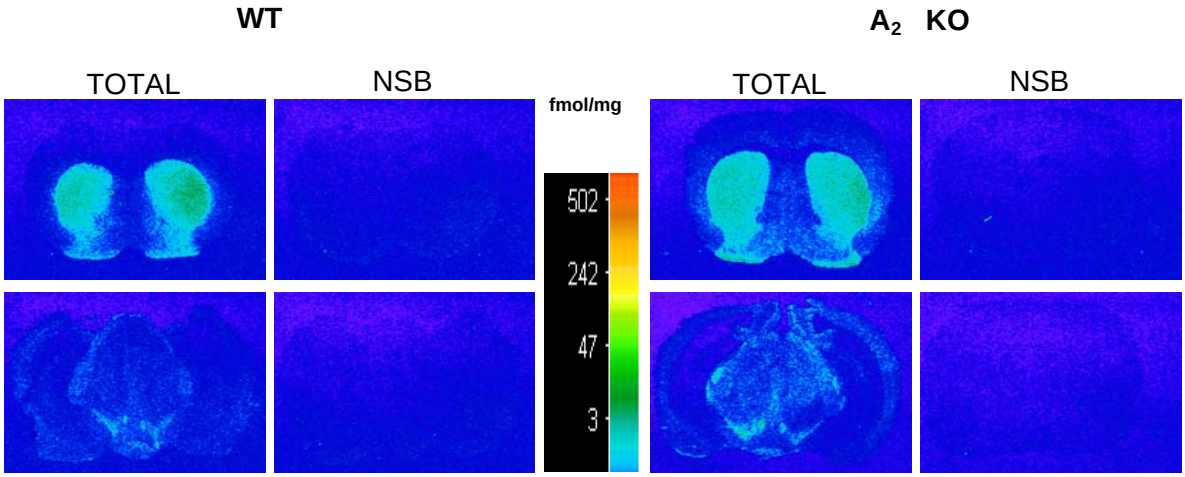

Table 1: Quantitative autoradiography of dopamine D_2-like, D_1-like receptors and dopamine transporters in brain regions of wild-type and adenosine A_{2A} receptor knockout mice

	Dopamine D_2 receptor		**Dopamine D_1-like receptor**		**Dopamine transporters**	
Brain Regions	**WT (fmol/mg)**	**A_{2A} KO (fmol/mg)**	**WT (fmol/mg)**	**A_{2A} KO (fmol/mg)**	**WT (fmol/mg)**	**A_{2A} KO (fmol/mg)**
Nucleus accumbens	53.5 ± 4.2	60 ± 3.5	237.2 ± 15.8	248.1 ± 14.3	266.9 ± 20.2	200 ± 89.6
Caudate putamen rostral	84.7 ± 6.5	91.6 ± 4.1	317.7 ± 10.8	347.7 ± 9.6	386.1 ± 18.9	312.7 ± 135.8
Caudate putamen caudal	80.7 ± 10.3	97.7 ± 5.9	220.7 ± 10.8	250.7 ± 9.6	261.6 ± 14.9	220.8 ± 41.9
Olfactory tubercle	50.4 ± 5.4	63.2 ± 9.0	253.6 ± 10.9	237.7 ± 17.1	277.1 ± 37.5	196.4 ± 77.1
Substantia nigra	13.6 ± 4.2	17.3 ± 6.4	146.4 ± 17.2	134.3 ± 5.8	149.6 ± 15.6	120.8 ± 27.7
Ventral tegmental area	7.6 ± 2.2	13.9 ± 5.2	12.5 ± 3.0	11.2 ± 2.0	187.7 ± 22.6	168.4 ± 20.0

Sections were labeled with 4 nM [^{3}H]raclopride (n=6), [^{3}H]SCH23390 (n=6) and [^{3}H]mazindol (n=4), for dopamine D_2-like and D_1-like receptors and transporters, respectively, in the wild-type (WT) and A_{2A} receptor knockout (KO) mice. Values are expressed as mean ± SEM. All structures were identified according to the brain atlas of Paxinos and Franklin (Paxinos & Franklin). Two-way ANOVA reveals significant genotype effect ($P < 0.05$) on overall D_2 receptor binding, without any significant differences in any single region in posthoc test ($P > 0.05$). D_1 receptors or dopamine transporter densities were not different between genotypes ($P > 0.05$, two-way ANOVA).