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Expression of D2 receptor isoforms in cultured neurons reveals equipotent autoreceptor function

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Abstract

Alternative splicing of the dopamine D2 receptor gene produces two distinct isoforms referred to as $D2_{long}$ (D2L) and $D2_{short}$ (D2S). In mesencephalic dopamine neurons, inhibition of the firing rate through activation of somatodendritic D2 receptors and blockade of neurotransmitter release through stimulation of terminal D2 receptors represent major roles of D2 autoreceptors. Recently, data obtained from D2L-deficient mice suggested that D2S acts as the preferential D2 autoreceptor. In the present study, we investigate whether this D2 isoform-specific autoreceptor function is linked to differences in the subcellular localization and/or signaling properties of the D2S and D2L using mesencephalic neurons transfected with enhanced green fluorescent protein (EGFP)-tagged receptors. Our results show that EGFP-tagged D2S and D2L are localized to the axonal and somatodendritic compartments of mesencephalic neurons. In addition, we demonstrate that EGFP-tagged D2S and D2L regulate cellular excitability, neurotransmitter release and basal levels of intracellular calcium with similar effectiveness. Overall, our morphological and electrophysiological studies suggest that the major D2 autoreceptor function attributed to D2S is likely explained by the predominant expression of this isoform in dopamine neurons rather than by distinct subcellular localization and signaling properties of D2S and D2L.

Keywords: Dopamine; D2 receptor; Firing; Presynaptic; Alternative splicing

1. Introduction

Deregulation of dopaminergic transmission has been implicated in a variety of neurological and psychiatric disorders including Parkinson's disease, schizophrenia, drug dependence and Tourette's syndrome (Missale et al., 1998). Dopamine acts through five transmembrane G-protein coupled receptors (GPCRs). The dopamine receptors can be divided in two subclasses: D1-like (D1 and D5) and D2-like (D2, D3 and D4) (Missale et al., 1998). The dopamine D2 receptor plays a key role in the regulation of dopaminergic transmission (Mercuri et al., 1997; L'hirondel et al., 1998). It acts as the primary autoreceptor leading to rapid inhibition of dopamine release (May and Wightman, 1989; Benoit-Marand et al., 2001).

Alternative gene splicing generates two distinct isoforms of the dopamine D2 receptor referred to as $D2_{long}$ (D2L) and $D2_{short}$ (D2S) (Bunzow et al., 1988; Dal Toso et al., 1989). D2L distinguishes itself from D2S only by the presence of a 29 amino acid insert within the third intracellular loop (Bunzow et al., 1988; Dal Toso et al., 1989). Because the third intracellular loop plays a central role in the coupling of GPCRs to their effectors, the existence of alternatively spliced D2 receptors imply a role for the 29 amino acid insert in the

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2, 3-dione; D2L, $D2_{long}$; D2S, $D2_{short}$; EGFP, enhanced green fluorescent protein; EPSC, excitatory postsynaptic current; GPCR, G-protein coupled receptor; IPSC, inhibitory postsynaptic current; MAP2, microtubule-associated protein-2; PKC, protein kinase; PMA, phorbol 12-myristate 13-acetate.

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functional diversity of the two isoforms. Although D2S and D2L share the same pharmacological profile (Leysen et al., 1993; Castro and Strange, 1993) recent studies performed in heterologous cell lines have evaluated the possibility that the two isoforms display distinct G protein coupling properties. In fact, studies have reported that D2S and D2L display differential abilities to regulate adenylyl cyclase activity and voltage-gated channels (Montmayeur et al., 1993; Senogles, 1994; Liu et al., 1994; Guiramand et al., 1995; Liu et al., 1996, 1999; Wolfe and Morris, 1999). Moreover, D2S and D2L are differently regulated by protein kinase C (PKC) (Liu et al., 1992; Liu et al., 1994; Choi et al., 1999). In light of these results, it is hypothesized that D2S and D2L may control different transduction pathways and physiological roles in neurons.

Recently, D2L knockout (D2L-KO) mice (Usiello et al., 2000) were used to investigate the functional role of the D2 receptor splice variants in vivo (Usiello et al., 2000; Wang et al., 2000). Inhibition of locomotor activity by low doses of D2 agonists, blockade of dopamine neuron firing by activation of somatodendritic receptors and reduction of dopamine release in response to activation of terminal D2 receptors are unchanged in D2L-KO in comparison to wild type mice. Because these agonist actions are mediated by autoreceptor activation, studies using D2L-KO mice have implicated D2S as the major dopamine autoreceptor. In support of this idea, haloperidol-induced catalepsy, which is mediated by a blockade of postsynaptic D2 receptors, is significantly reduced in D2L-KO mice suggesting an important postsynaptic role for D2L. However, it remains unclear whether the isoform-specific presynaptic and postsynaptic roles are due simply to a preferential splicing in dopamine neurons leading to higher expression of D2S (Khan et al., 1998), or whether D2S exhibits distinctive functional characteristics allowing this isoform to mediate autoreceptor function. In addition, it is important to consider separately the somatodendritic and terminal autoreceptor functions of the D2 receptor. The specific implication of D2S and D2L in autoreceptor function in these two compartments has not been directly explored. However, both isoforms appear to contribute to the somatodendritic effects of D2 agonists on GABAergic neurons of the striatum (Centonze et al., 2004).

In this report, rat primary mesencephalic neurons transfected with enhanced green fluorescent protein (EGFP)-tagged D2 receptor isoforms were used to determine whether D2S and D2L are differentially targeted to somatodendritic and axonal compartments and coupled to various functional effectors. We find that D2S and D2L can be localized to somatodendritic and axonal compartments. Moreover, our studies show that they are equipotent in inhibiting neuronal firing and neurotransmitter release.

2. Methods

2.1. Plasmids

pEGFP-N3 was obtained from BD Biosciences Clontech (Palo Alto, CA, USA). Enhanced green fluorescent protein (EGFP)-tagged human D2S and D2L expression constructs were prepared in pCMV5. Briefly, the DNA sequence coding for a modified version of a cleavable signal peptide (M-K-T-I-A-L-S-Y-I-F-C-L-V-F-A) fused to EGFP was introduced at the N-terminus of the D2S and D2L isoforms using an overlap PCR-based methodology (Sedaghat, Nantel, Ginsberg, Lalonde and Tiberi, submitted).

2.2. Cell culture

Primary cultures of rat mesencephalic neurons were prepared from Sprague–Dawley rats (P0–P2) as previously described (Cardozo, 1993; Sulzer et al., 1998; Michel and Trudeau, 2000; Bourque and Trudeau, 2000; Congar et al., 2002). Dissociated neurons were plated on mesencephalic astrocytes grown in monolayers on pre-coated glass coverslips. For single neurons micro-cultures, dissociated cells were plated onto astrocyte micro-islands. Cultures were maintained in Neurobasal-A/B27 medium (Gibco) supplemented with penicillin/streptomycin, GlutaMAX-1 (Gibco) and 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA). Among the cell type present in these mesencephalic cultures, dopaminergic neurons account for 25–30% of neurons.

2.3. Transfection

Cultured neurons grown for 2 days on coverslips were transferred from their home dish to a 12-well plate (Corning, NY, USA) with 1 ml of Neurobasal-A/B27 medium (Gibco) supplemented with penicillin/streptomycin and GlutaMAX-1 (Gibco). Neurons were transfected using a modified Ca^{2+}-phosphate transfection protocol (Shieh et al., 1998). Four micrograms of DNA was used for each coverslip. In some experiments co-transfection with the pEGFP-N3 plasmid (EGFP alone) was done to lower the expression of D2 receptor isoforms. In such cases, the total amount of DNA was maintained constant while the ratio of EGPP-D2L or EGFP-D2S to EGFP alone was 1:10. This assured that the decrease in receptor level was not accompanied by a decrease in transfection efficiency. After transfection, neurons were cultured in their original medium at 37 °C and 5% CO_2 for 4–8 days. To determine whether the expression of each plasmid was comparable after transfection in cultured neurons, we quantified the intensity of EGFP fluorescence in unfixed living transfected neurons as an index of D2 receptor expression. The signal intensity, evaluated using identical acquisition parameters, was not different in neurons expressing EGFP-D2S ($n = 40$) in comparison to neurons expressing EGFP-D2L ($n = 29$) (t-test, $P = 0.76$) (not shown).

2.4. Immunocytochemistry

Transfected cultured neurons were fixed with 4% paraformaldehyde in phosphate-buffered solution (pH 7.4) and incubated with a polyclonal anti-GFP antibody (BD Sciences Clontech) or a polyclonal anti-D2 antibody (150 mg/mL, final dilution 1:250) (Maltais et al., 2000) to detect the overexpressed EGFP-D2R fusion proteins. The detection of transfected D2 receptors using an anti-GFP (100 mg/mL, final dilution 1:250) antibody allowed a more sensitive detection of receptor level and localization since we found that after fixation, the intrinsic intensity of EGFP fluorescence was greatly reduced. Monitoring endogenous EGFP fluorescence was however effective to identify transfected neurons before electrophysiological recordings. For immunocytochemistry, the anti-GFP or anti-D2 primary antibodies were visualized using an Alexa-488-labeled secondary antibody (2 mg/mL, final dilution 1:200) (Molecular Probes Inc., Eugene, OR, USA). Monoclonal anti-MAP2 (9.7 mg/mL, final dilution 1:250) (Sigma) or anti-Ankyrin B antibodies (0.5 mg/mL, final dilution 1:250) (Zymed Laboratories Inc., San Francisco, CA, USA), detected with an Alexa-546-labeled secondary antibody (2 mg/mL, final dilution 1:200), were used to identify dendritic structures or axons, respectively. In some experiments triple labeling was performed, using in addition a sheep anti-tyrosine hydroxylase antibody (0.2 mg/mL, final dilution 1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to identify dopamine neurons. This primary antibody was visualized with a Cy5-labeled secondary antibody (1.5 mg/mL, final dilution 1:200) (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). Images of immunofluorescent labeling were acquired using a Hamamatsu Orca-II digital cooled CCD camera and

an Inovision workstation using Isee software (Inovision Corporation, Raleigh, NC, USA). Additional immunofluorescence images were acquired using a point scanning confocal microscope from Prairie Technologies LLC (Middletown, WI, USA). Excitation was achieved using the 488 nm line of an argon ion laser and with the 633 nm line of a helium-neon laser.

2.5. Electrophysiology

Electrophysiological recordings were performed at room temperature on transfected neurons maintained for 6–10 days in culture. The standard extracellular bathing solution contained (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 6 sucrose, 10 glucose, pH 7.35, ≅300 mOsm. Drugs were bath applied, with a delay between valve opening and onset of drug action of approximately 15 s. Whole-cell current clamp recordings were performed using a Warner PC-505 patch-clamp amplifier (Warner Instruments Corp., Hamden, CT, USA). Signals were filtered at 1 kHz, digitized at 10 kHz and recorded and analyzed using Pclamp7 software (Axon Instruments, Foster City, CA, USA) and Mini Analysis software (version 5.6) (Synaptosoft Inc., Leonia, NJ, USA) respectively. Action potentials were recorded with borosilicate glass patch pipettes (5–7 MΩ) filled with a potassium methylsulfate intrapipette solution containing (mM): 145 $KMeSO_4$, 20 KCl, 10 NaCl, 0.1 EGTA, 2 ATP (Mg salt), 0.6 GTP (Tris salt), 10 HEPES, 10 phosphocreatine (Tris salt) (pH 7.35). In all experiments, the firing frequency was normalized to the average frequency recorded in the baseline period (2 min before drug application) and expressed as a percentage. The effect of a drug on firing rate was determined as the ratio of mean firing frequency at the peak of the effect (usually 1 min for the effect of the D2 agonist quinpirole) to the average frequency measured during baseline and washout periods. This calculation method takes into account any rundown in firing rate that could occur during long recording periods.

Synaptic responses in single neurons were recorded using the ruptured whole-cell patch-clamp technique. These synaptic responses are generated by synapses (or ''autapses'') established by the neurons' axon terminals onto its own dendrites. During whole-cell recording, autaptic responses were evoked every 15 s by a brief (1 ms) depolarizing voltage step from a holding potential (V_H) of −50 mV. In excitatory neurons, this usually elicited a sodium ''action current'' followed by a 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)-sensitive glutamate-mediated inward autaptic EPSC. In inhibitory neurons, autaptic currents evoked by brief (1 ms) depolarizing voltage steps from a holding potential (V_H) of −40 mV, displayed reversal potentials close to −50 mV and were sensitive to 5 μM SR-95531, a γ-aminobutyric acid-A ($GABA_A$) receptor antagonist.

Mesencephalic cultures used contain dopaminergic neurons, expressing D2 receptors endogenously. We therefore performed post-hoc immunofluorescence after each electrophysiological recording to identify neuronal phenotype. Dopaminergic neurons constituted a minority of the recorded neurons (less than 25%). Since the magnitude of responses mediated by endogenous D2 receptors in these cells is much smaller than in neurons overexpressing the D2 receptor-mediated responses (not shown) and the responses measured in D2-transfected dopaminergic neurons was not different from those observed in D2 transfected non-dopaminergic neurons, all experiments were pooled. In addition, although dopaminergic neurons in culture have the ability to release dopamine, the extracellular levels during our experiments should have been minimal considering that cells were continuously perfused with saline. The activation of transfected D2 receptors by endogenous dopamine should not have affected our results. Finally, it should be pointed out that non-dopaminergic neurons in mesencephalic cultures are unresponsive to D2-like receptor agonists (Congar et al., 2002).

2.6. Calcium imaging

Changes in cytosolic intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured with Fura 2 ratio fluorescence. Briefly, cells were loaded with fura 2 by incubating cells grown on 15-mm coverslips in saline (pH 7.4) containing 5 μM fura-2-AM and 0.02% pluronic acid (Molecular Probes, Eugene, OR, USA) for 45 min at room temperature. For $[Ca^{2+}]_i$ measurements, the coverslip was mounted in a recording chamber that was fixed to the stage of an inverted Nikon Eclipse TE-200 microscope. The coverslip was constantly perfused with physiological saline solution (containing in mM: NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, glucose 10, sucrose 6 at a pH of 7.35), this solution was maintained at 37 °C. $[Ca^{2+}]_i$ was measured by digital fluorescence imaging using Ratiotool software (Inovision Corp., Raleigh, NC, USA). Fluorescence excitation at 340/380 nm was controlled by a DG4 xenon lamp (Sutter Instruments, Novato, CA, USA). Fluorescence was collected after passing through a 495 nm long pass filter. Excitation image pairs (340/380 nm) were acquired every 5 s with a Orca-II digital cooled-CCD camera (Hamamatsu, Bridgewater, NJ, USA). $[Ca^{2+}]_i$ was calculated using an in situ calibration protocol and Grynkiewicz's equation (Grynkiewicz et al., 1985). We considered a neuron non-responsive when a change of less than 50nM in $[Ca^{2+}]_i$ was measured in response to a pharmacological agent.

2.7. Statistical analysis

The results are expressed as arithmetic means ± S.E.M. For statistical analysis, Two-Factor ANOVA and *t*-tests were used as indicated.

3. Results

3.1. Subcellular localization of dopamine D2S and D2L receptors

The ligand binding affinities and coupling to the inhibition of adenylyl cyclase of EGFP-tagged D2S and D2L receptors were similar and not impaired when compared with their wild-type counterparts using heterologous expression in human embryonic kidney 293 cells (Sedaghat et al., submitted). All subsequent experiments were performed in postnatal rat mesencephalic neurons in primary culture. Expression of EGFP-D2 fusion proteins was detected by immunocytochemistry using an anti-D2 antibody (Fig. 1C–E). This signal could not be mistaken with endogenous D2 receptor expression in mesencephalic neurons because D2 receptor abundance is low in cultured dopaminergic neurons and in fact undetectable by immunocytochemistry (Fig. 1A,B). However, EGFP-D2L (Fig. 1C) and EGFP-D2S (Fig. 1D) fusion proteins are easily detectable in cultured transfected neurons. Because high quality anti-D2 antibodies are not readily available and because the expression profile of EGFP-D2 fusion proteins is quite similar when detected using anti-D2 (Fig. 1E) or anti-GFP antibodies (Fig. 1F), all subsequent immunolabeling experiments were performed using a anti-GFP antibody. To characterize the subcellular expression pattern of each splice variants, double-labeling was performed using an antibody directed against MAP2, to identify dendritic structures (Caceres et al., 1986), or ankyrin B (Zhang and Bennett, 1998) to identify axons. EGFP-D2L (Fig. 2A–C) and EGFP-D2S (Fig. 2D–F) co-localized with MAP2, suggesting that both isoforms can be targeted to the somatodendritic compartment. In addition to the somatodendritic expression, EGFP-D2S and EGFP-D2L receptors were also observed in small caliber processes that did not express MAP2 protein, which suggest that the two isoforms could also be targeted to a subcellular compartment reminiscent of axons (Fig. 2G). Overall, the axonal-like expression of D2 receptors was observed in 45% and 36% of neurons expressing EGFP-D2S and EGFP-D2L, respectively (Table 1) (Chi-square = 0.686, $P > 0.05$). To confirm that

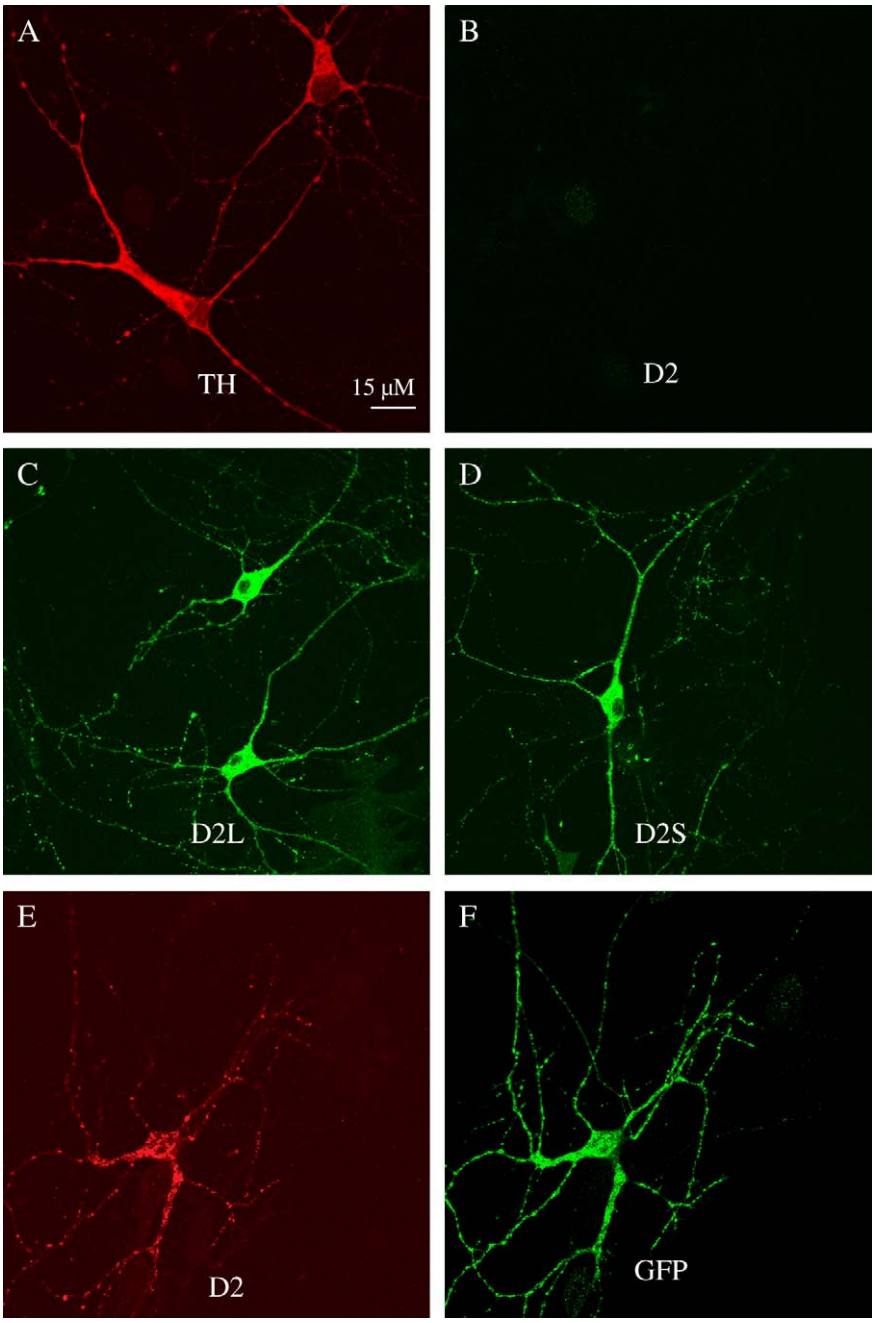

Fig. 1. Overexpression of D2 receptor isoforms in cultured mesencephalic neurons. Confocal immunofluorescence images of midbrain cultured neurons. These cultures contain dopaminergic neurons as detected by anti-tyrosine hydroxylase (TH) antibodies (A). Although dopamine neurons endogenously expressed D2 receptors these receptors are expressed at levels too low to be detected using an anti-D2 antibody (B). Immunofluorescence images of midbrain cultured neurons transfected with EGFP-D2L (C) or with EGFP-D2S (D–F). Cellular expression of D2 receptor fusion proteins (green) can both be detected using an anti-D2 antibody (C–E) and an anti-GFP antibody (F). As can be seen, the expression profile looks quite similar when the same transfected neuron was double-immunostained for D2 (E) or GFP (F). Scale bar in A: 15 µm.

these small MAP2-negative processes expressing EGFP-D2 fusion receptors were indeed axons, double-labeling was performed using an antibody against ankyrin B, a protein only found in axons (Zhang and Bennett, 1998) (Fig. 2H,I). As a positive control for our ability to detect EGFP-labeled axons, we overexpressed the cytoplasmic form of EGFP alone in neurons. The majority of transfected neurons displayed a thin MAP2-negative process expressing EGFP (81%, Table 1).

Using a triple-labeling approach, we next evaluated whether receptor targeting was different in dopaminergic neurons in comparison to non-dopaminergic neurons in the same cultures. Tyrosine hydroxylase immunolabeling was used to identify dopaminergic neurons, while EGFP-D2S and EGFP-D2L were detected using the anti-GFP antibody. The expression profile of EGFP-D2S and EGFP-D2L was not different in dopaminergic neurons compared to non-dopaminergic neurons.

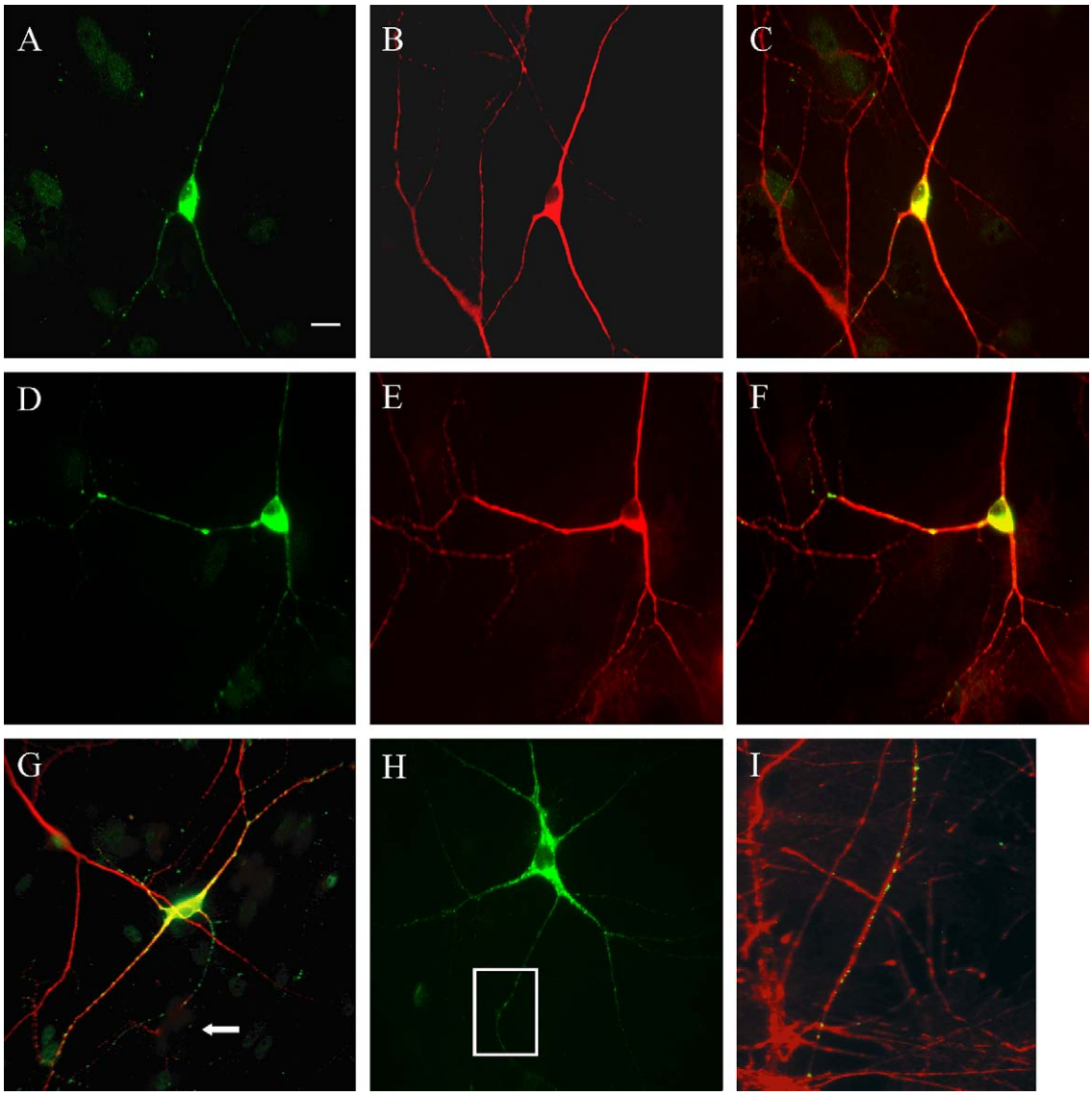

Fig. 2. Subcellular localization of D2S and D2L dopamine receptors. Immunofluorescence images of cultured neurons transfected with EGFP-D2L (A–C) or with EGFP-D2S (D–H). Cellular expression of D2 receptor fusion proteins (green) was detected using an anti-EGFP antibody (A,D,H). Both D2S and D2L co-localize with the dendritic marker MAP2 (red) (B–E) as shown by the overlapping signals (yellow/orange) (C,F). The immunofluorescence image in G illustrates the localization of EGFP-D2S (green) and the dendritic marker MAP2 (red). The neuron shows a small caliber process that is immuno-negative for MAP2 (white arrow). The image shown in H illustrates another neuron expressing EGFP-D2S (green). The enlargement (I), originating from the area indicated by the white box in H, shows a double-labeling image illustrating the co-localization of D2S (green) and the axonal marker ankyrin B (red). Scale bar in A: 15 μm.

Indeed, expression of EGFP-D2S was observed in small caliber MAP2-negative processes (presumed axons) in 55% ($n = 20$) of transfected dopaminergic neurons and in 42% ($n = 39$) of non-dopaminergic neurons (Chi-square = 0.553, $P > 0.05$). Likewise, expression of EGFP-D2L was observed in presumed axons in 50% ($n = 16$) of transfected dopaminergic neurons and in 31% ($n = 38$) of non-dopamine transfected neurons (Chi-square = 0.944, $P > 0.05$) (not shown).

3.2. D2S and D2L are equipotent in inhibiting firing rate

We next tested the hypothesis that the preferential autoreceptor role of the D2S might result from an enhanced capacity to inhibit firing frequency. Whole-cell recordings were obtained from neurons transfected with EGFP-D2L or EGFP-D2S (Fig. 3A). Quinpirole (1 μM), a D2-selective agonist, caused a reduction in the spontaneous firing rate and membrane hyperpolarization in EGFP-D2S and EGFP-D2L transfected neurons (Fig. 3B,C). The quinpirole-induced decrease in firing did not show pronounced acute desensitization and was stable during the 5 min agonist applications (Fig. 3B,C). A comparison of summary data shows that both isoforms were equipotent to inhibit firing rate (paired t-test, $P > 0.05$) (Fig. 3D). A comparison of maximal effects showed that activation of EGFP-D2S by quinpirole (1 μM) caused an inhibition of $74.9 \pm 4.0\%$ ($n = 14$) in firing frequency while an inhibition of $66.4 \pm 6.9\%$ ($n = 15$) was observed in

Table 1
Localization of D2 receptor isoforms in transfected neurons

	Proportion of neurons showing a EGFP+/MAP2− process (%)	No. of neurons showing a EGFP+/MAP2− process	Total transfected neurons
EGFP-D2S	36	27	59
EGFP-D2L	45	20	55
EGFP	81	30	37

The first column indicates the proportion of neurons transfected with either EGFP-D2S, EGFP-D2L or EGFP alone, in which a presumed axon (MAP2-negative) contained detectable EGFP signal. The second and third columns provide raw data including the total number of transfected neurons detected by immunocytochemistry using anti-GFP antibodies. The identification of a process as EGFP-positive and MAP2-negative (thus not a dendrite) was unequivocal considering the robust level of MAP2 immunostaining.

neurons expressing EGFP-D2L. A closer examination of the kinetics of the effect of quinpirole on spontaneous firing in 9 cells revealed that the time required to reach half of the maximal effect was quite similar in neurons expressing EGFP-D2S or EGFP-D2L (35 ± 9.6 s and 46.1 ± 7.8 s, *t*-test $P > 0.05$). The half-time of recovery had a tendency to be more rapid with EGFP-D2S, but the difference was not statistically significant (210.8 ± 49.4 s and 107.7 ± 31.2 s, *t*-test $P = 0.1$).

The aforementioned set of experiments was made using a saturating dose of quinpirole (1 μM) to assess response desensitization. Additionally, a dose–response relationship for quinpirole-mediated inhibition of spontaneous firing frequency in neurons expressing EGFP-D2S or EGFP-D2L receptors

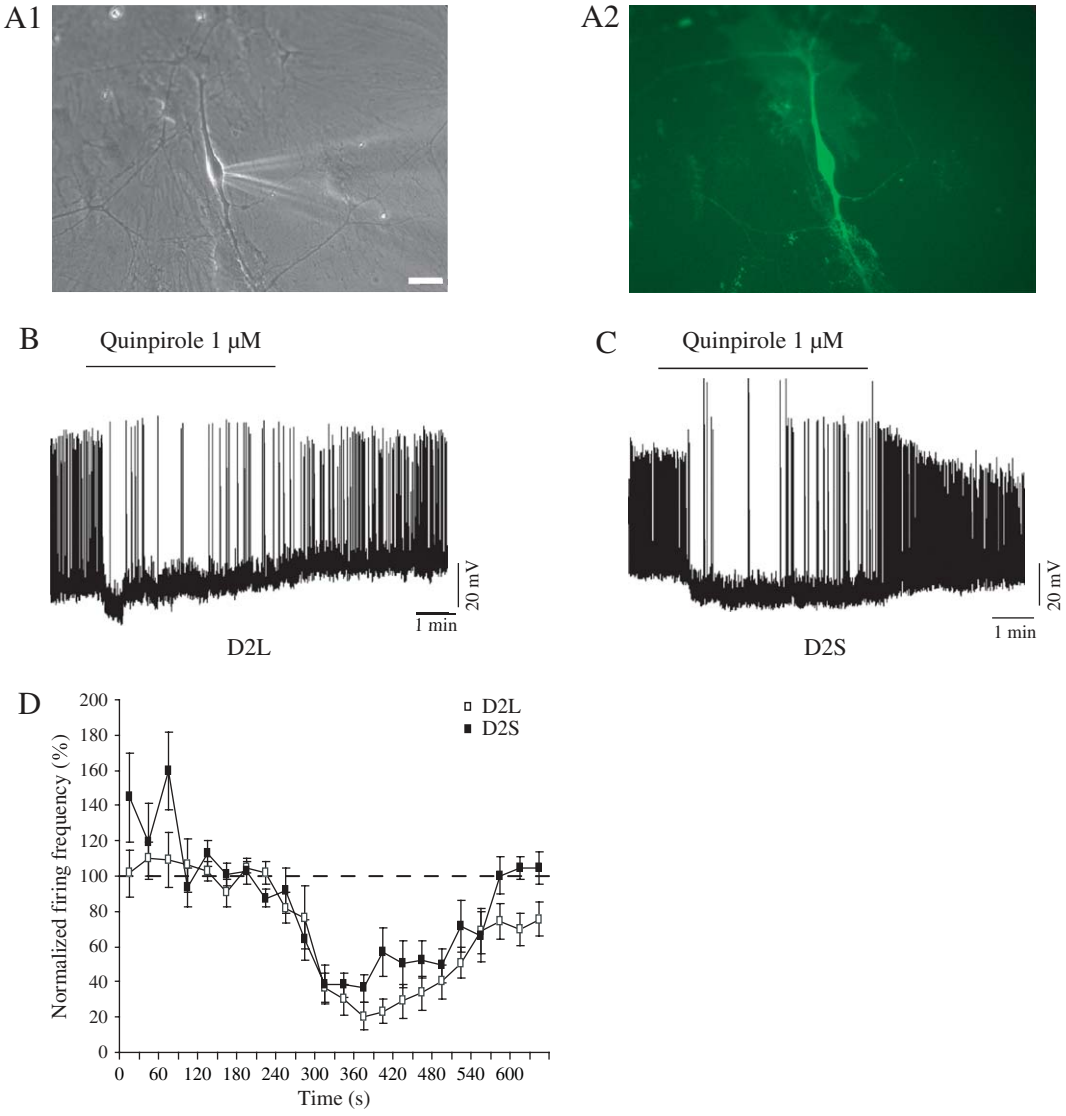

Fig. 3. D2S and D2L are equipotent in inhibiting firing rate. (A1) Phase contrast image of a patched EGFP-D2S transfected neuron (A2). Scale bar: 20 μm. (B,C) Representative examples of patch-clamp recordings of spontaneous action potentials in neurons transfected with EGFP-D2L (B) or EGFP-D2S (C). Quinpirole (1 μM; 5 min; black bar) caused a strong reduction of firing frequency. (D) Graph summarizing experiments performed on neurons transfected with EGFP-D2L (□) ($n = 9$) and EGFP-D2S (■) ($n = 9$). Data are expressed as mean ± S.E.M.

was made (Fig. 4A,B). No statistically significant differences between the two groups were detected (two-way ANOVA; $P > 0.05$ for the effect of the isoform; $P < 0.01$ for the effect of the dose) (Fig. 4A,B).

As a control, the ability of the D2 antagonist sulpiride (5 μM) to block the effect of quinpirole was evaluated in a subset of neurons. For these experiments, quinpirole (1 μM) was first applied alone for 2 min and, after a 6 min wash out period, it was applied again in the presence of sulpiride (5 μM). In these neurons, the maximal inhibition of firing frequency induced by the activation of EGFP-D2S or EGFP-D2L with quinpirole was $78.1 \pm 9.5\%$ ($n = 4$) and $74.4 \pm 11.7\%$ ($n = 5$), respectively, while this inhibition was reduced to $-3.7 \pm 15.8\%$ and $9.2 \pm 10.8\%$, respectively, in the presence of sulpiride (paired t-test, $P < 0.001$ and $P < 0.05$, respectively) (Fig. 4C).

3.3. D2S and D2L receptor isoforms can act presynaptically to inhibit neurotransmitter release

In dopaminergic neurons, release can also be directly modulated through terminal D2 autoreceptors. Considering our immunolabeling results demonstrating that both D2S and D2L proteins can be efficiently targeted to axons, we wondered whether both receptor isoforms can also act at axon terminals to inhibit neurotransmitter release. To answer this question we transfected single isolated neurons, a preparation in which synaptic terminals are established onto the neuron's own dendrites, thus facilitating measurements of action potential-evoked synaptic transmission.

During whole-cell recording, a brief (1 ms) depolarizing voltage step evoked a fast inward sodium current (generating

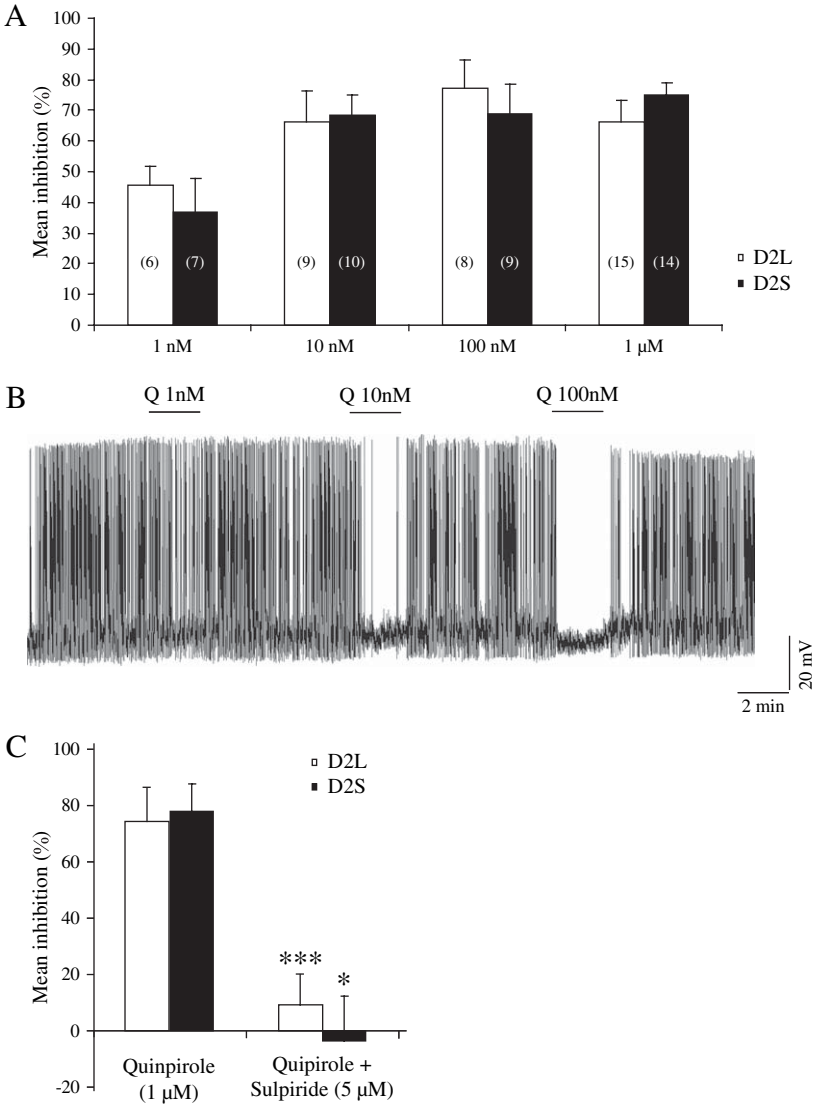

Fig. 4. Dose–response curve of the effect of the D2 agonist quinpirole on the firing rate of transfected neurons. (A) Histogram showing the dose-response relationship of quinpirole on firing frequency. The number of observations is indicated in parentheses for each group. The columns represent the maximal inhibition of firing rate induced by quinpirole. (B) Representative example of a patch-clamp recording experiment measuring spontaneous action potentials in neurons transfected with EGFP-D2L. Increasing concentrations of quinpirole (1, 10 and 100 nM) were applied for 2 min each and separated by a 6 min wash out period. (C) Sulpiride (5 μM) blocked the effect of Quinpirole (Quin) on firing frequency. The graph represents the maximal inhibition of firing rate induced by quinpirole with or without sulpiride. EGFP-D2L (□) ($n = 5$) and EGFP-D2S (■) ($n = 4$). Data are expressed as mean ± S.E.M. *$P < 0.05$, ***$P < 0.001$.

an unclamped action potential) followed by a postsynaptic, CNQX-sensitive excitatory postsynaptic current (autaptic EPSC) (Fig. 5A,B) in the majority of neurons. In a few neurons, the evoked postsynaptic current was sensitive to the $GABA_A$ receptor antagonist SR95531 and was thus a GABAergic IPSC (not shown). Bath application of quinpirole (1 μM) robustly and reversibly decreased the peak amplitude of autaptic synaptic currents in isolated neurons expressing EGFP-D2L (Fig. 5A) or EGFP-D2S (Fig. 5B). Activation of EGFP-D2L by quinpirole caused a $69.6 \pm 6.2\%$ inhibition of autaptic postsynaptic current amplitude ($n = 7$, Student t-test $P < 0.01$) (Fig. 5C). The magnitude of inhibition was similar in neurons expressing EGFP-D2S ($66.8 \pm 6.2\%$, $n = 5$, Student t-test $P < 0.05$) (Fig. 5D). An additional set of experiments was performed to evaluate whether expressing lower amounts of receptors would reveal a differential targeting to the axon and differential ability to act as presynaptic receptors. We cotransfected neurons with EGFP-D2S or EGFP-D2L and pEGFP-N3 plasmids (EGFP alone) at a ratio of 1:10. We found that when using a ten-fold lower quantity of D2 plasmid, quinpirole decreased the amplitude of autaptic postsynaptic currents by $65.5 \pm 7.9\%$ ($n = 7$, $P \leq 0.006$) in neurons expressing EGFP-D2L while the decrease was of $62.8 \pm 4.9\%$ ($n = 5$, $P \leq 0.007$) in neurons expressing EGFP-D2S (not shown). This finding confirms our previous morphological and physiological data showing that EGFP-D2S and EGFP-D2L both reach the axonal compartment and act as terminal autoreceptors in an equivalent manner.

3.4. D2S and D2L receptor isoforms do not induce calcium mobilization

Although D2 receptor stimulation has been found to mobilize intracellular calcium in certain cell lines (Vallar et al., 1990; Tang et al., 1994; Ghahremani et al., 1999) and in striatal medium spiny neurons (Hernandez-Lopez et al., 2000), this ability has been recently proposed to require co-activation of D1 dopaminergic receptors (Lee et al., 2004). However, it remains undetermined whether D2S or D2L expressed alone in neurons can influence intracellular calcium. To evaluate this possibility, transfected cultured neurons were loaded with fura-2 AM, a fluorescent calcium indicator, and changes in $[Ca^{2+}]_i$ were measured with fura-2 ratio fluorescence. We found that neither D2S nor D2L isoforms cause an elevation of intracellular calcium levels, even in response to relatively high doses of quinpirole (5 μM). In fact, activation D2S or D2L isoforms by quinpirole caused a decrease in basal

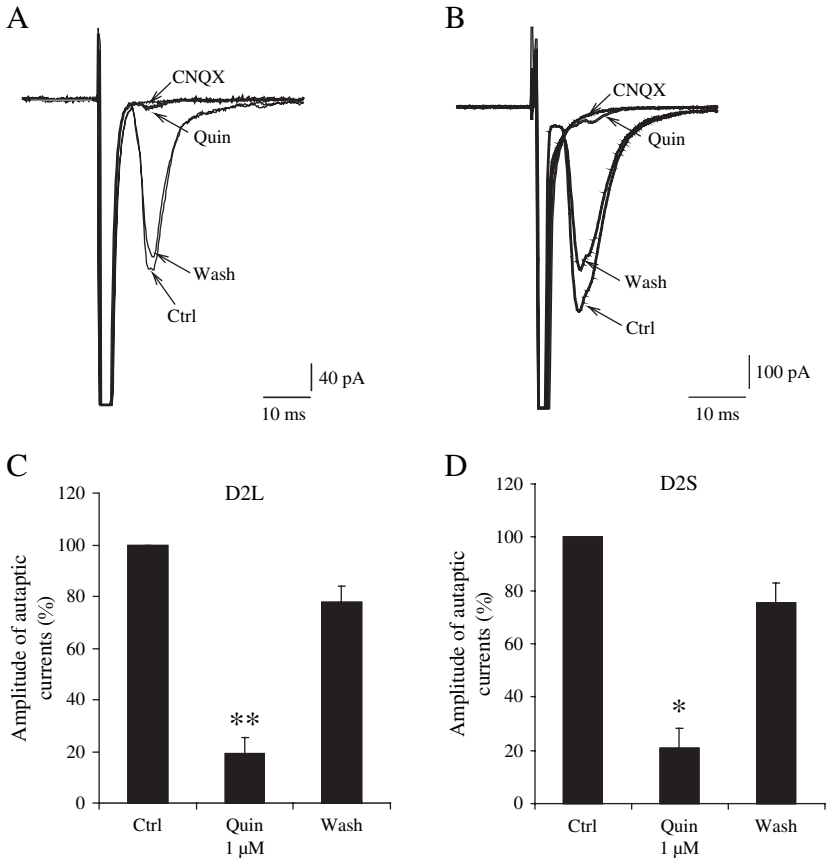

Fig. 5. Both D2S and D2L mediate presynaptic inhibition in single transfected neurons. (A,B) Patch-clamp recordings of EPSCs evoked by single action potentials in isolated neurons transfected with EGFP-D2L (A) or EGFP-D2S (B). In excitatory neurons, the autaptic response corresponds to a CNQX-sensitive glutamate-mediated inward autaptic EPSC. Quinpirole (Quin) (1 μM) caused a strong reduction of the amplitude of the autaptic response. (C,D) Summary graph of the effect of quinpirole (Quin) on the amplitude of autaptic responses in neurons transfected with EGFP-D2L ($n = 7$) (C) or EGFP-D2S ($n = 5$) (D). The amplitude of autaptic responses was normalized to that observed during the baseline period. Data are expressed as mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$.

intracellular calcium levels (32.7 ± 6.8% and 31.9 ± 6.7% respectively, paired *t*-test $P < 0.001$) (Fig. 6A,B). The effect of quinpirole was similar in D2S-EGFP and D2L-EGFP transfected neurons (Fig. 6C) (*t*-test, $P > 0.05$). All transfected neurons showed an increase in intracellular calcium in response to membrane depolarization using 40 mM K^+-containing saline (not shown), used to assess cell viability.

4. Discussion

In the present study, we expressed EGFP-tagged dopamine D2 receptor isoforms in primary cultured neurons to compare their relative efficacy as somatodendritic and terminal receptors. We found that D2S and D2L are equipotent in the regulation of neuronal excitability and neurotransmitter release, two well-known functions associated with somatodendritic and terminal D2 autoreceptors, respectively. These results are consistent with the subcellular localization observed for each isoform. Indeed, we found that transfected neurons expressed EGFP-D2S and EGFP-D2L in their axons in addition to their somatodendritic compartment.

In light of the recent studies using D2L-KO mice (Usiello et al., 2000; Wang et al., 2000; Lindgren et al., 2003), it has been suggested that D2 receptor isoforms could serve distinct

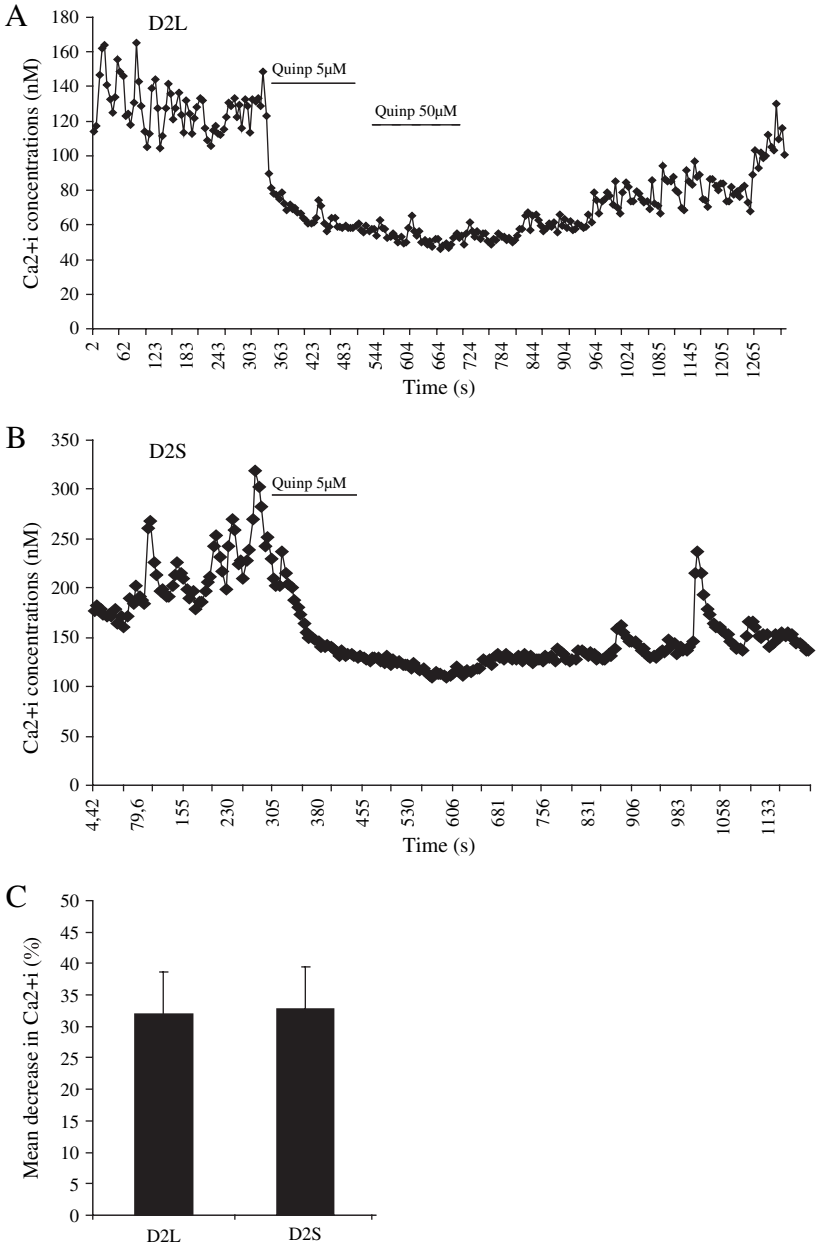

Fig. 6. Both D2S and D2L decrease intracellular calcium concentration. (A,B) Example of fura-2 calcium imaging experiments in rat cultured neurons transfected with EGFP-D2L (A) or EGFP-D2S (B). Quinpirole (5 and 50 µM) was applied for 5 min. Transfected neurons were identified prior the experiment by epifluorescence. (C) Summary graph of the effect of quinpirole (Quin) on basal intracellular [Ca^{2+}] in neurons transfected with EGFP-D2L ($n = 12$) or EGFP-D2S ($n = 13$). Data are normalized to baseline period and expressed as mean ± S.E.M.

physiological functions. These studies indicate that the short isoform is the major D2 dopamine autoreceptor while the D2L plays mostly a postsynaptic role. This idea is further supported by a study showing that the D2S is more abundantly expressed in dopaminergic neurons (Khan et al., 1998). Considering that dopamine neurons can also express D2L, we wished to evaluate whether any functional differences between D2S and D2L explains the preferential function of D2S as autoreceptor in dopaminergic neurons. When overexpressed in primary cultured neurons, EGFP-tagged dopamine D2S receptors did not show a better efficiency than D2L receptors to act as somatodendritic and terminal autoreceptors. This lack of difference persisted even when using low agonist concentrations or when using 10-fold lower plasmid levels for transfection, consistent with the idea that differences were not masked due to exceedingly high receptor numbers (although we cannot completely exclude that small differences were masked by receptor overexpression). These results help to clarify previous results on D2 receptor isoform expression in dopaminergic neurons by suggesting that the reason for preferential D2S autoreceptor function in dopaminergic neurons is quantitative rather than qualitative, i.e. D2S functions as the major autoreceptor in dopaminergic neurons because preferential splicing leads to more abundant D2S and not because D2L has some diminished capacity to function as a terminal or somatodendritic autoreceptor. Our results therefore do not contradict previous studies implicating the D2S isoform in autoreceptor function in the midbrain (Usiello et al., 2000; Joseph et al., 2002; Centonze et al., 2002; Rouge-Pont et al., 2002). Although in another system, it is also interesting to note that D2S and D2L equally participate in the somatodendritic inhibitory effect of D2 agonists on GABAergic neurons of the striatum (Centonze et al., 2004).

The D2 subtype of dopamine receptor represents the main autoreceptor within the dopaminergic system (Mercuri et al., 1997; L'hirondel et al., 1998; Usiello et al., 2000), but is also critical for postsynaptic transmission. Since it has been previously shown that stimulation of D2 receptors in acutely isolated striatal neurons (Hernandez-Lopez et al., 2000) or in D2 receptor-transfected GH4C1 or CCL1.3 cells (Vallar et al., 1990; Tang et al., 1994; Ghahremani et al., 1999), can cause an increase in intracellular calcium, we were interested to evaluate the ability of the D2L dopamine receptor, the isoform associated with postsynaptic functions, to couple to intracellular calcium mobilization. In contrast to these studies, our data clearly show that neither D2L nor D2S activation promotes calcium mobilization in neurons under our experimental conditions, even though we used relatively high doses of D2 agonist (see Khan et al., 2001 for calcium mobilization in astrocytes with high doses of quinpirole). In fact, the basal intracellular calcium level was diminished to a similar extent by activation of each D2 receptor isoforms, a phenomenon that likely results from the inhibition of firing rate, leading to reduced activation of voltage-gated calcium channels. A direct inhibition of voltage-gated calcium channels by the D2 receptor could also be involved (Cardozo and Bean, 1995). The fact that we see no intracellular calcium mobilization in response to a D2 receptor agonist is in agreement with the recent suggestion that co-activation of D1 and D2 receptors is required for calcium mobilization (Lee et al., 2004). Co-expression experiments in cultured neurons would be useful to extend this conclusion to neurons.

The dopamine D2 receptor is the first GPCR for which a splice variant was reported (Monsma et al., 1989). Since this discovery, studies of the functional consequences of alternative splicing have focused on both ligand binding and signaling efficacy. Until now, attempts to examine differential coupling of D2 dopamine receptor isoforms to G-protein subunits have led to conflicting results (Senogles, 1994; Liu et al., 1994; Guiramand et al., 1995). In addition, the differences reported between D2S and D2L in their coupling efficiency are likely to depend on the expression system. For example, while in HEK293 cells EGFP-D2L appears to be less readily expressed at the plasma membrane than EGFP-D2S (Sedaghat et al., submitted), when expressed in cultured neurons, we see no difference in the ability of these same receptors to be activated by a D2 agonist leading to firing rate inhibition. It was thus important to closely evaluate possible differences in coupling using intact neurons as a model. Despite these discrepancies, compelling evidence indicates that D2S is the preferential autoreceptor while D2L acts mainly at postsynaptic sites in vivo. In the present study, we showed that in transfected neurons, both isoforms are equipotent to regulate neuronal excitability as well as neurotransmitter release. Our findings support the hypothesis that D2S is the major autoreceptor in dopaminergic neurons because of its higher expression levels in these cells (Khan et al., 1998) rather than because it displays some unique properties for the coupling to the rapid signaling pathways that mediate the inhibition of neurotransmitter release and cellular excitability in axon terminals and somatodendritic compartment, respectively.

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