The Brain Cytoplasmic RNA BC1 Regulates Dopamine D₂ Receptor-Mediated Transmission in the Striatum

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Dopamine D₂ receptor (D₂DR)-mediated transmission in the striatum is remarkably flexible, and changes in its efficacy have been heavily implicated in a variety of physiological and pathological conditions. Although receptor-associated proteins are clearly involved in specific forms of synaptic plasticity, the molecular mechanisms regulating the sensitivity of D₂ receptors in this brain area are essentially obscure. We have studied the physiological responses of the D₂DR stimulations in mice lacking the brain cytoplasmic RNA BC1, a small noncoding dendritically localized RNA that is supposed to play a role in mRNA translation. We show that the efficiency of D₂-mediated transmission regulating striatal GABA synapses is under the control of BC1 RNA, through a negative influence on D₂ receptor protein level affecting the functional pool of receptors. Ablation of the BC1 gene did not result in widespread dysregulation of synaptic transmission, because the sensitivity of cannabinoid CB₁ receptors was intact in the striatum of BC1 knock-out (KO) mice despite D₂ and CB₁ receptors mediated similar electrophysiological actions. Interestingly, the fragile X mental retardation protein FMRP, one of the multiple BC1 partners, is not involved in the BC1 effects on the D₂-mediated transmission. Because D₂DR mRNA is apparently equally translated in the BC1-KO and wild-type mice, whereas the protein level is higher in BC1-KO mice, we suggest that BC1 RNA controls D₂DR indirectly, probably regulating translation of molecules involved in D₂DR turnover and/or stability.

Key words: electrophysiology; plasticity; IPSC; GABA transmission; noncoding RNA; mRNA localization

Introduction

Dopamine (DA) regulates the output of striatal neurons through the stimulation of D₁-class (D₁, D₅) and D₂-class (D₂, D₃, D₄) receptors. Ordered DA transmission in this brain area is essential for the control of motor activity, reward, and cognition (Berke and Hyman, 2000; Obeso et al., 2000), and alterations of DA D₂ receptor (D₂DR) signaling are implicated in neurological and psychiatric conditions. For example, reduced sensitivity of striatal D₂DR occurs in a mouse model of familial Parkinson's disease (Goldberg et al., 2005), whereas increased sensitivity seems to mediate striatal neuron dysfunction and degeneration in Huntington's disease (Charvin et al., 2005). Furthermore, reduced D₂DR activity in the striatum has been implicated in the maintenance of cocaine self-administration (Nader et al., 2006), and increased sensitivity of these receptors plays a role in schizophrenia (Hunt et al., 2005) and anxiety (Ponnusamy et al., 2005).

Localized mRNA/RNAs control local synthesis of cytosolic, cytoskeletal, receptor, and receptor-associated proteins and might be important for the modulation of neurotransmission (Steward and Schuman, 2003). Among these RNA molecules, the noncoding brain cytoplasmic BC1 RNA is highly abundant in dendritic and axonal domains of cortical and hippocampal neurons (Muslimov et al., 2002; Nimmrich et al., 2005; Johnson et al., 2006). BC1 RNA interacts with several RNA binding proteins, including Staufen (Mallardo et al., 2003), the testis–brain protein (Muramatsu et al., 1998), purine-rich binding proteins (Pur α and Pur β) (Kobayashi et al., 2000), poly(A) binding protein (PABP) (Muddashetty et al., 2002), and the fragile X mental retardation protein (FMRP) (Zalfa et al., 2006). Since its evolutionary conservation with alanine tRNA (Rozhdestvensky et al., 2001), BC1 has been hypothesized to be involved in mRNA translation. BC1 knock-out (KO) mice have been generated recently (Skryabin et al., 2003) and showed reduced exploration and increased anxiety (Lewejohann et al., 2004).

To shed light on the potential role of the small neuronal noncoding RNA in the control of DA-mediated transmission, we studied the physiological responses of striatal neurons to the

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stimulation of D₂ receptors in mice lacking the BC1 RNA (BC1-KO). Our data indicate that BC1 RNA negatively controls the sensitivity of D₂ receptors at striatal GABAergic synapses and that increased functional pool of the D₂ receptors is detectable in the BC1-KO mice. Because D₂DR protein level is increased in the striatum of the BC1-KO mice and D₂DR mRNA translational efficiency is unaltered, we propose that the observed effect is probably attributable to a different turnover and/or stability of D₂DR protein in the absence of BC1 RNA.

Materials and Methods

Transgenic animals. Male mice lacking BC1 RNA [BC1-KO, generated in 129SV × C57BL/6 strain (Skryabin et al., 2003)] or FMRP [FMR1-KO, generated in C57BL/6 strain (Bakker et al., 1994)] were used along with their age-matched wild-type (WT) counterparts (2–3 months old).

Animal care was conducted conforming to the institutional guidelines that are in compliance with national (Italian law DL N116) and international laws and policies (European Community Council Directive 86/609; National Institutes of Health *Guide for the Care and Use of Laboratory Animals*).

Electrophysiology. Corticostriatal coronal slices (200 μ m) were prepared from tissue blocks of the mouse brain with the use of a vibratome (Centonze et al., 2005). A single slice was then transferred to a recording chamber and submerged in a continuously flowing artificial CSF (ACSF) (32°C, 2–3 ml/min) gassed with 95% O₂–5% CO₂. The composition of the control solution was as follows (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 2.4 CaCl₂, 11 glucose, and 25 NaHCO₃.

Recording pipettes were advanced toward individual striatal cells under visual control and positive pressure, and, on contact, tight gigohm seals were made by applying negative pressure. The membrane patch was then ruptured by suction, and membrane current and potential were monitored using an Axopatch 1D patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Whole-cell access resistances measured in voltage clamp were in the range of 5–20 M Ω .

Whole-cell patch-clamp recordings were made with borosilicate glass pipettes (1.8 mm outer diameter; 2–5 M Ω) at the holding potential of –80 mV. To detect GABA_A-mediated evoked (eIPSCs) and miniature (mIPSCs) IPSCs, intracellular solution had the following composition (in mM): 110 CsCl, 30 K⁺-gluconate, 1.1 EGTA, 10 HEPES, 0.1 CaCl₂, 4 Mg-ATP, and 0.3 Na-GTP. MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate] (30 μ M) and CNQX (10 μ M) were added to the external solution to block, respectively, NMDA and non-NMDA glutamate receptors. For synaptic stimulation, intra-striatal bipolar electrodes were used. eIPSCs were evoked at a frequency of 0.1 Hz.

mIPSCs were stored by using pClamp 9 (Molecular Devices) and analyzed off-line on a personal computer with Mini Analysis 5.1 (Synaptosoft, Leonia, NJ) software. The detection threshold was set at twice the baseline noise. The fact that no false events would be identified was confirmed by visual inspection for each experiment. Off-line analysis was performed on mIPSCs recorded during a fixed time epoch (2–3 min), sampled every 2–3 min before (four to five samplings) and after (5–10 samplings) the application of quinpirole. Only cells that exhibited stable frequencies in control (<20% changes during the control samplings) were taken into account. For kinetic analysis, events with peak amplitude between 10 and 50 pA were grouped, aligned by half-rise time, and normalized by peak amplitude. Events with complex peaks were eliminated. In each cell, all events between 10 and 50 pA were averaged to obtain rise times, decay times, and half-widths.

Drugs were applied by dissolving them to the desired final concentration in the bathing ACSF. The concentrations of the various drugs were chosen according to previous *in vitro* studies on brain slices and were as follows: CNQX (10 μ M), GR 103691 (4'-acetyl-N-[4-[4-(2-methoxyphenyl)-1-piperazinyl]butyl]-[1,1'-biphenyl]-4-carboxamide) (5 μ M), HU210 [(6aR,10aR)-3-(1,1'-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol] (1 μ M), MK-801 (30 μ M), and tetrodotoxin (TTX) (1 μ M) were from Tocris (Bristol, UK); bicuculline (10 μ M), L-sulpiride (3 μ M), quinpirole (0.3–10

μ M), and R(+)-7-hydroxy-DPAT hydrobromide (1 μ M) were from Sigma (St. Louis, MO).

Semiquantitative reverse transcription-PCR. RNA from WT and BC1-KO mouse brains (entire and striatum) was extracted using Trizol (Invitrogen, Carlsbad, CA). RNA quantity and quality were assessed by measuring UV absorbance at 260 nm and by electrophoresis on agarose gel, respectively. Total RNA was DNase treated (GE Healthcare, Little Chalfont, UK) and reverse transcribed as described previously (Zalfa et al., 2003). An aliquot (2 μ l) of the reverse transcription (RT) reaction was amplified by PCR in a final volume of 50 μ l, using 20 pmol of each primer, 0.5 U of TaqDNA polymerase (GE Healthcare), 100 mM dTTP, dATP, and dGTP, 10 mM dCTP, and 0.2 mCi of [α -³²P]dCTP (3000 Ci/mmol; GE Healthcare).

The amount of template and the number of amplification cycles were preliminarily optimized for each PCR reaction to avoid saturation: 22 cycles for the D₂DR mRNA, 14 cycles for the β -actin mRNA. Radioactive PCR products were run on a 5% polyacrylamide gel and quantified by PhosphorImager (Molecular Dynamics). D₂DR mRNA levels were then normalized to β -actin mRNA levels and expressed as arbitrary units. For each animal, three semiquantitative RT-PCRs were performed. A total of three animals were analyzed.

Polysomal analysis. The analysis was performed as described previously (Zalfa et al., 2003). Briefly, total brain and the entire striatum was removed from the whole brain and homogenized in lysis buffer (100 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM DTT, 40 U/ml RNase inhibitor, 10 μ l/ml protease inhibitor cocktail, 5 mM β -glycerophosphate, and 0.5 mM Na-orthovanadate). After 5 min of incubation on ice, the homogenates were centrifuged at 12,000 rpm at 4°C for 5 min. The supernatant was quickly frozen in liquid nitrogen and stored at –80°C until usage. Cytoplasmic extracts were separated a 15–50% (w/v) sucrose gradient by centrifugation for 110 min at 37,000 rpm in a Beckman Coulter (Fullerton, CA) SW41 rotor. Each gradient was collected in 10 fractions. RNA was extracted from each gradient fraction and analyzed by radioactive RT-PCR using the primers listed below. To correct for variations in the efficiency of the RT-PCR reaction, the same amount of a synthetic RNA (L22; this RNA was obtained by *in vitro* transcription of the *Xenopus* ribosomal protein L22 sequence) was added to each fraction after the gradient and before the RNA extraction, amplified, and used for normalization. Products were run on a 5% polyacrylamide gel and quantified by a PhosphorImager.

Antibodies. The following primary antibodies were used in this study: mouse monoclonal D₂DR (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), β -actin (1:15,000; Sigma), mouse monoclonal anti-microtubule-associated protein 2 (MAP2; 1:100; Sigma), mouse monoclonal anti-tau (1:1000; Chemicon, Temecula, CA), mouse monoclonal anti-GAD-65 (1:100; Sigma), mouse monoclonal anti-parvalbumin (1:100; Chemicon), rabbit polyclonal anti-GABA (1:500; Sigma), and anti-digoxigenin rhodamine conjugated (1:300; Roche, Indianapolis, IN). The secondary antibodies used were as follows: anti-mouse and anti-rabbit secondary antibodies conjugated to fluorophores [cyanine 2 (Cy2)/Cy3/Cy5; 1:100; Jackson ImmunoResearch, West Grove, PA] and anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:10,000; Promega, Madison, WI).

Western blotting. BC1-KO and WT mice were processed for Western blotting. The entire brain (BC1-KO, $n = 6$; WT, $n = 4$) and the striatum area (BC1-KO, $n = 7$; WT, $n = 5$) were homogenized in lysis buffer (50 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X-100, 10% glycerol, and 320 mM sucrose containing 10 μ l/ml Sigma protease inhibitor). Thirty micrograms of total extracts were separated by SDS-PAGE and probed with antibodies against D₂DR and β -actin, followed by secondary antibodies conjugated to horseradish peroxidase and developed with the ECL-plus (GE Healthcare). Images were acquired by a Storm 840 (GE Healthcare), and quantification was performed by using ImageQuant (version 5.0 TL v2003.02; Molecular Devices). D₂DR levels were then normalized to β -actin and expressed as arbitrary units.

Binding of [³⁵S]GTP γ S. Striata (BC1-KO, $n = 6$; WT, $n = 6$) were homogenized in 50 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, and 3 mM MgCl₂ and centrifuged at 1000 $\times g$ for 10 min. Supernatants were further centrifuged for 10 min at 49,000 $\times g$. The resulting membrane

Figure 1. Effects of D₂DR stimulation on evoked IPSCs in corticostriatal slices of WT and BC1-KO mice. **A**, Quinpirole reduced eIPSC amplitude in both WT (white circles) and BC1-KO (black circles) striatal neurons. Traces below are examples of voltage-clamp recordings showing the effects of quinpirole on eIPSCs recorded from WT and BC1-KO mice. **B**, The depressant effect of quinpirole was dose dependent and remarkably potentiated in BC1-KO mice compared with WT mice for 1–10 μM concentrations. **C**, R(+)-7-hydroxy-DPAT hydrobromide, selective agonist of D₂DRs, failed to reduce eIPSC amplitude in both WT and BC1-KO mice. **D**, Preincubation with GR 103691, a selective D₂DR antagonist, failed to prevent the effects of quinpirole on eIPSCs recorded from both WT and BC1-KO striatal neurons. **E**, Quinpirole application enhanced PPR in WT and BC1-KO mice. Right shows examples of PPR recordings before and during the application of 10 μM quinpirole. **p* < 0.05; ***p* < 0.01.

fractions were washed, resuspended in the same buffer, and centrifuged twice for 10 min at 49,000 × *g*. The pellet was finally resuspended in the assay buffer (in mM: 50 Tris-HCl, pH 7.4, 0.2 EDTA, 3 MgCl₂, and 100 NaCl), divided into aliquots, and stored at –80°C. Membrane preparations were first preincubated in 4 mU/ml adenosine deaminase (183 U/mg of protein; Sigma). The binding of [³⁵S]GTPγS (0.1 nM, 1250 Ci/mmol; PerkinElmer, Wellesley, MA) on quinpirole-stimulated membranes (20 μg) was performed in the presence of 10 μM GDP in the assay buffer for 1 h at 30°C (Geurts et al., 1999; Gainetdinov et al., 2003). The antagonist of D₂DR (L-sulpiride, 3 μM; Sigma) was preincubated for 10

min before the assay. Nonspecific binding was measured in the presence of 30 μM GTPγS. Incubations were terminated by rapid filtration of samples through glass filter (GF/C; Whatman, Clifton, NJ).

Striatal cell culture. Striatal cell cultures were obtained from postnatal day 2 mice. The brains were removed from the pups and freed of meninges, and the striatum from each hemisphere was isolated and then treated with papain at 20 U/ml (+L-cysteine at 1 mM) for 15 min at 37°C. After the blockage of papain activity, using trypsin inhibitor, cells were resuspended in Neurobasal–2% B27 and gently triturated. The cell suspension was centrifuged, resuspended in the same medium, and plated on a poly-lysine-coated dishes. Cultures were maintained at 37°C for 7 d *in vitro* (DIV) before usage.

Preparation of cRNA probe. BC1 RNA was cloned into pGemTeasy vector starting from total brain RNA with primers described below. Linearization with SacII or with SacI and transcription with SP6 or T7 polymerases will lead to antisense or sense digoxigenin-labeled cRNA probes. RNA *in situ* hybridization was performed using antisense and sense probes for BC1 RNA. Specificity of the probe was assessed performing Northern blot analysis on WT and BC1-KO mice (data not shown).

Tissue preparation for fluorescence in situ hybridization and immunofluorescence. Mice were anesthetized and then perfused with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed and postfixed for 1 d in 4% paraformaldehyde/PBS. Brains were placed in 30% sucrose in 0.1 M PBS, embedded in the OCT compound (Sakura, supplied by Bio-Optica, Milan, Italy), and frozen on dry ice. Frozen brains were cryosectioned (20 μm thickness).

Fluorescence in situ hybridization and immunofluorescence on striatal neurons. Striatal neurons were fixed at room temperature for 15 min with 4% paraformaldehyde in 1 × PBS. Fluorescence *in situ* hybridization (FISH) and immunofluorescence were performed as described previously (Ferrari et al., 2007). Briefly, neurons were prehybridized in 50% formamide, 2 × SSC and 10 mM NaH₂PO₄ and hybridized overnight at 37°C in 30% formamide, 10 mM NaH₂PO₄, 10% dextran sulfate, 2 × SSC, yeast tRNA, and salmon sperm DNA, in the presence of *in vitro* synthesized digoxigenin-UTP antisense or sense BC1 RNA. After several washes in 2 × SSC–0.1% Triton X-100 and 1 × SSC–0.1% Triton X-100, neurons were incubated with primary and anti-digoxigenin rhodamine-conjugated antibodies. Primary and secondary antibodies were used at concentrations described above. The images were acquired using

a confocal microscope (LSM510; Zeiss, Oberkochen, Germany).

Oligonucleotides. The following oligonucleotides were used in this study: mouse D₂DR (producing two different fragments, 579 and 492 bp; GenBank accession number NM_010077), upstream, 5'-GGC CAT GCC TAT GTT GTA TAA-3'; downstream, 5'-CCC ATT CTT TTC TGG TTT GG-3'; mouse β-actin (fragment size, 575 bp; GenBank accession number NM_0073931), upstream, 5'-AGC AAG AGA GGT ATC CTG ACC-3'; downstream, 5'-GCC AAT AGT GAT GAC CTG GCC-3'; mouse BC1 (GenBank accession number U01310), upstream, 5'-GTT GGG GAT

TTA GCT CAG TGG-3'; downstream, 5'-AGG TTG TGT GTG CCA GTT ACC-3'; L22 (this RNA was obtained by *in vitro* transcription of the *Xenopus* ribosomal protein L22 sequence; GenBank accession number X64207.1); upstream, 5'-CGT GGG CAC GTC AGT CAC G-3'; downstream, 5'-TCG AGG TCG ACG GTA TC-3'.

Statistical analysis. For data presented as the mean $\pm$ SEM, statistical analysis was performed using a paired or unpaired Student's *t* test or Wilcoxon's test for comparison between two groups. One-way ANOVAs for independent measures were used for multiple comparisons. The significance level was established at $p < 0.05$. To determine whether two cumulative distributions of spontaneous synaptic activity were significantly different, the Kolmogorov–Smirnov test was used.

Results

Effects of D₂DR stimulation on eIPSCs in WT and BC1-KO mice

The intrinsic membrane properties of the recorded neurons were similar in BC1-KO and WT mice and did not differ from those reported previously for spiny neurons of the striatum (Centonze et al., 2005) (data not shown). After intrastriatal stimulation, GABA-mediated eIPSCs were recorded, in the presence of MK-801 plus CNQX, in both WT and BC1-KO mice. These currents were fully blocked by the GABA_A receptor antagonist bicuculline (data not shown).

In neurons from WT mice, application of quinpirole (0.3–10 μ M, 10 min; $n = 10$ for each dose), agonist of the D₂ class of dopamine receptors, significantly reduced eIPSCs (0.3 μ M: $p < 0.05$, $t = 2.60$; 1 μ M: $p < 0.01$, $t = 4.29$; 3 μ M: $p < 0.001$, $t = 7.10$; 10 μ M: $p < 0.001$, $t = 6.93$), an effect prevented or reversed by preincubating the slices with the selective antagonist of D₂ class of dopamine receptors L-sulpiride ($n = 4$; $p > 0.05$ for both groups). In neurons from BC1-KO mice, the effects of quinpirole were remarkably potentiated ($n \geq 9$ for each concentration, 0.3 μ M: $p < 0.05$, $t = 2.91$ with respect to predrug values and $p > 0.1$, $t = 0.71$ compared with WT; 1 μ M: $p < 0.001$, $t = 6.71$ with respect to predrug values and $p < 0.05$, $t = 2.48$ compared with WT; 3 μ M: $p < 0.0001$, $t = 8.93$ with respect to predrug values and $p < 0.001$, $t = 4.29$ compared with WT; 10 μ M: $p < 0.0001$, $t = 9.21$ with respect to predrug values and $p < 0.001$, $t = 4.43$ compared with WT), and L-sulpiride fully prevented ($n = 4$; data not shown) or reversed the effects of quinpirole also in these mutants ($n = 5$; $p > 0.05$) (Fig. 1*A,B*).

Previous experiments in mice lacking D₂DR have indicated that the effects of quinpirole on striatal GABA transmission are entirely mediated by this subclass of DA receptors (Centonze et al., 2004). Increased sensitivity to quinpirole in BC1-KO mice, however, does not necessarily imply changes in the sensitivity of D₂DRs, because other members of the D₂ class of dopamine receptors might be involved. To address this issue, we initially mea-

Figure 2. Effects of D₂DR stimulation on striatal mIPSCs in WT and BC1-KO mice. **A**, Quinpirole application reduced mIPSC frequency recorded in the presence of TTX in neurons from WT (white circles) and BC1-KO (black circles) mice. The depressant effect of quinpirole was potentiated in BC1-KO mice and was washed out by bath application of D₂ receptor antagonist L-sulpiride. **B**, The graph shows the change of cumulative distributions of mIPSC interevent interval during the application of quinpirole in WT mice. **C**, Example of a voltage-clamp recording showing the reduction of mIPSC frequency caused by D₂ receptor stimulation in a WT striatal neuron. **D**, The graph shows the change of cumulative distributions of mIPSC interevent interval during the application of quinpirole in BC1-KO mice. **E**, Example of a voltage-clamp recording showing the reduction of mIPSC frequency caused by D₂ receptor stimulation in a BC1-KO striatal neuron.

sured the effects of R(+)-7-hydroxy-DPAT hydrobromide, selective agonist of D₃DRs, in both WT and BC1-KO mice. This compound (10 min incubation) caused negligible effects on eIPSC amplitude in both WT and BC1-KO mice ($n = 9$ and $p > 0.05$ for both groups) (Fig. 1*C*). Furthermore, preincubation of striatal slices in the presence of the D₃DR antagonist GR 103691 (10 min) failed to prevent the effects of 3 μ M quinpirole on eIPSCs recorded from both WT and BC1-KO striatal neurons ($n = 5$ for both groups; $p > 0.05$ compared with quinpirole alone) (Fig. 1*D*).

To investigate whether the effects of D₂DR stimulation on striatal GABA transmission were presynaptic, we measured the action of quinpirole (10 μ M) on paired-pulse ratio (PPR) of IPSCs evoked with an interstimulus interval of 70 ms. In both WT ($n = 6$) and BC1-KO ($n = 9$) mice, the depressant effect of quinpirole on eIPSCs was associated with a significant increase in

Figure 3. BC1 RNA localization in striatal axons. **A–C**, Immunofluorescence for Tau (**A**) and MAP2 (**C**) coupled with FISH for BC1 RNA (**B**) on primary striatal cells at 7 DIV. White arrows in **B** indicate the BC1 RNA staining in axonal processes that are not stained for the dendritic marker MAP2. **D–F**, Immunofluorescence for Tau (**D**) and FISH for BC1 RNA (**E**) on brain slices (striatum); **F** is the merged image. White arrows point areas of colocalization (yellow). **G–I**, Immunofluorescence for GAD-65 (**G**) and FISH for BC1 RNA (**H**) on primary striatal cells at 7 DIV and on the striatum (**J–L**). **L** is the merged image. White arrows point areas of colocalization of BC1 RNA and GAD-65. Scale bars, 10 μ m.

PPR (eIPSC₂/eIPSC₁), as expected for a presynaptic action of the drug ($p < 0.01$ for both groups; $t = 3.49$ for WT and $t = 4.28$ for BC1-KO) (Fig. 1E).

Effects of quinpirole on striatal mIPSCs in WT and BC1-KO mice

In line with the results on eIPSCs, quinpirole (10 μ M) also reduced GABAergic mIPSC frequency in slices from WT and BC1-KO mice (7–10 min) ($n = 10$ and $p < 0.05$ for both groups). In slices from BC1-KO mice, the effect of quinpirole was significantly greater ($p < 0.01$, $t = 4.45$) (Fig. 2A–E). Preincubation (5–10 min) with the D₂DR antagonist L-sulpiride prevented the effects of quinpirole on mIPSCs in both WT and BC1-KO mice ($n = 5$ and $p > 0.05$ for both groups; WT, $98 \pm 3.2\%$ of pre-quinpirole values; BC1-KO, $102 \pm 4.0\%$ of pre-quinpirole values). mIPSC mean amplitude (WT, $n = 10$, $99 \pm 3.5\%$ of pre-quinpirole values; BC1-KO, $n = 10$, $97 \pm 4.5\%$ of pre-quinpirole values), rise time, decay time, and half-width (data not shown)

were unaffected by quinpirole ($n = 10$ and $p > 0.05$ for both groups and each parameter).

Specificity of the BC1 RNA–D₂DR interaction

Some of the physiological activities of BC1 RNA depend on the interaction with the FMRP (Zalfa et al., 2006). To test whether FMRP–BC1 RNA interaction was also involved in the BC1 RNA-dependent modulation of D₂DR signaling, we performed electrophysiological recordings in mice lacking the FMR1 gene. In FMR1-KO striatal neurons and in their respective WT cells, the depressant action of quinpirole (10 μ M) on eIPSCs was similar ($n = 8$ for both groups; WT, $84 \pm 4.6\%$ of pre-quinpirole values; FMR1-KO, $82 \pm 7.0\%$ of pre-quinpirole values; $p > 0.05$) (data not shown).

Other receptors participate in the pre-synaptic modulation of GABA transmission in the striatum, including the cannabinoid CB₁ receptors (Centonze et al., 2005). Thus, we investigated whether the abnormal control on GABA synapses observed in the BC1-KO mice was specific for the D₂DR. Application of the cannabinoid CB₁ receptor agonist HU210 (10 min; $n = 7$) in neurons from WT animals significantly ($p < 0.05$) reduced eIPSCs ($78 \pm 4.6\%$ of pre-HU210 values). In neurons from BC1-KO mice ($n = 7$), HU210 produced comparable effects ($80 \pm 4.6\%$ of pre-HU210 values; $p > 0.05$) (data not shown).

BC1 RNA is localized in striatal axons

The electrophysiological transmission impairment observed in the BC1-KO mice implies the action of BC1 RNA in striatal axons, in which it could play a direct or indirect role on D₂DR function. First, we verified the BC1 RNA presence in axons of

striatal neurons performing FISH conjugated to immunofluorescence in striatal cell culture as well as in brain sections from the striatum.

BC1 RNA was present in neurites of striatal neurons lacking the specific dendritic marker MAP2 (Aronov et al., 2002) and expressing the axonal marker Tau (Binder et al., 1985), showing its expression in axons (Fig. 3B, white arrows). FISH analysis performed in striatal brain sections (Fig. 3D–F) showed, by its colocalization with Tau, a specific axonal staining for BC1 RNA. Finally, thus the majority of striatal neurons are GABAergic, we checked, through the GAD-65 presence, a specific marker of GABAergic axons (Kaufman et al., 1991), the BC1 RNA presence in these neurons. In both striatal cell culture (Fig. 3G–I) and in the whole striatum (Fig. 3J–L), BC1 RNA partially colocalized with GAD-65.

We also investigated whether BC1 RNA was present in striatal interneurons, which represent an important source of GABA inputs to striatal projection cells. As shown in Figure 4, BC1 RNA

was present in parvalbumin-positive GABAergic interneurons (Fig. 4D, white arrow) as well as in parvalbumin-negative GABAergic neurons, possibly representing medium spiny projection cells (Fig. 4D, white arrowhead).

D₂DR expression and activity in the striatum of the BC1-KO mice

It has been reported that BC1 RNA plays a role in specific neuronal mRNA translation (Zalfa et al., 2006) and can also function as translation repressor in reticulocyte extracts (Wang et al., 2002). We decided to investigate whether the observed hypersensitivity of striatal D₂DR signaling in the BC1 mutants was the consequence of a dysregulation (increase) of the D₂DR. At first, we analyzed the D₂DR mRNA and protein levels. The steady state of D₂DR mRNA in total brain did not present any difference between WT and BC1-KO mice (Fig. 5A). Interestingly, both the long (D₂L) and short (D₂S) D₂DR isoforms showed the same amplification pattern (Fig. 5A,C). The D₂DR protein level detected in total brain from both WT and BC1-KO (Fig. 5B) was comparable although a not significant tendency to increase was detected in the BC1-KO mice ($n = 5$). Interestingly, when the same analysis was performed on the isolated striatum, the D₂DR mRNA levels were still comparable (Fig. 5C) ($p > 0.05$), but the D₂DR protein was remarkably increased ($n = 7$; $p < 0.01$) (Fig. 5D). β -Actin mRNA and protein (Fig. 5A–D) as well as glyceraldehyde-3-phosphate dehydrogenase (data not shown) did not show significant modulation, indicating specificity for the D₂DR.

Finally, to provide evidence that the increase in D₂DR level observed in BC1 RNA knock-out mice is correlated with an increase in the functional response, we measured the binding of GTP γ S induced by quinpirole from striatal membranes. As shown in Figure 5E, the binding activity observed with this assay was higher in the BC1-KO mice compared with WT mice after quinpirole stimulation ($n = 6$ experiments; $p < 0.05$). Interestingly, this effect was reduced in the presence of a DA D₂-class receptor antagonist L-sulpiride (data not shown).

To investigate whether the increased expression level of D₂DR in BC1-KO striatum was dependent on a different translational efficiency of D₂DR mRNA in WT and BC1-KO striatum, we analyzed its distribution between actively translating polysomes and silent messenger ribonucleoproteins (mRNPs). Quantification demonstrated that D₂DR mRNA (both isoforms) was translated with the same efficiency in total brain and in the striatum from BC1-KO and WT mice (Fig. 6A,B). Because BC1 has been shown to act as a translational inhibitor at the initiation level (Wang et al., 2002), we concluded that an impairment of translation at post-initiation phase can be excluded.

Discussion

BC1 RNA is part of a ribonucleocomplex involved in the regulation of protein synthesis in neurons (Zalfa et al., 2006). BC1 is efficiently transported along dendrites and axons (Muslimov et al., 2002) and acts as a translational repressor of chimeric mRNAs (Wang et al., 2002). A role for BC1 RNA as a mediator of dendritic mRNA transport (Muslimov et al., 1997) and as a crucial binding partner for the FMRP and other proteins has also been proposed (West et al., 2002; Mallardo et al., 2003; Johnson et al., 2006; Zalfa et al., 2006).

Despite the putative involvement of BC1 RNA in the control

Figure 4. BC1 RNA localization in striatal GABAergic neurons. **A–D**, FISH for BC1 RNA (**A**) coupled with fluorescence for GABA (**B**) and parvalbumin (**C**) on primary striatal cells at 7 DIV. The white arrowhead in (**D**, Merge) indicates a parvalbumin-negative GABAergic neuron, and the white arrow indicates a parvalbumin-positive GABAergic cell. Scale bar, 10 μ m.

Figure 5. D₂DR mRNA and protein expression levels in total brain and striatum. **A, C**, Top, Semiquantitative RT-PCR analysis of D₂DR mRNA on total brain (**A**) and striatal (**C**) RNA extracted from WT and BC1-KO mice (only 1 sample for each group is shown). Bottom, Histograms represent the sum of long and short isoform normalized for β -actin ($n = 3$). **B, D**, D₂DR protein levels in total brain (**B**) and striatum (**D**) from WT and BC1-KO mice. Top, Western blot detecting D₂DR and β -actin proteins (only 1 sample for each group is shown). Bottom, D₂DR protein expression level normalized for β -actin (WT, $n = 5$; BC1-KO, $n = 7$). **E**, [³⁵S]GTP γ S binding to striatal membranes from WT and BC1-KO was determined after stimulation with quinpirole. The data are expressed as percentage stimulation over basal and represent the mean $\pm$ SD (WT, $n = 3$; BC1-KO, $n = 3$). * $p < 0.05$.

of synaptic transmission, so far no study has addressed its role at the physiological level. Using BC1-KO mice, here we have showed that the efficiency of D₂DR-mediated transmission in this brain area is under the control of BC1 RNA, through a pro-

Figure 6. D₂DR mRNA partition between polysomes and mRNPs. **A, B.** Total brain (**A**) and striatal extracts (**B**) from WT (left) and BC1-KO (right) mice were fractionated by sucrose gradient centrifugation. Radioactive signals in the polysomal and nonpolysomal fractions were quantified, corrected versus the control RNA (L22), and expressed as percentage messenger on polysome (PMP). A typical polysomal profile from total brain (**A**) and striatum (**B**) in WT (left) and BC1-KO (right) extracts is shown. One of the RT-PCR experiments performed to amplify L22 RNA, β -actin, and D₂DR mRNAs is shown below. Histograms to the right represent the percentage messenger on polysome. Values shown are the means $\pm$ SEM ($n = 3$).

cess likely mediated by a negative influence on D₂DR insertion, turnover, and/or stability. Because BC1 RNA is a regulator of translation *in vitro* but BC1 RNA ablation affects neither mRNA abundance nor translation efficiency of the D₂DR mRNA *in vivo*, it is conceivable that BC1 RNA controls D₂DR activity indirectly. Our data showing that BC1 RNA was present in axons of GABAergic neurons of the striatum support the concept that this small RNA does regulate the expression level and function of D₂DRs at the presynaptic level, because the D₂DR-mediated inhibition of striatal GABA transmission is presynaptically mediated (Centonze et al., 2004).

Striatal GABAergic principal neurons, besides inhibiting basal ganglia output nuclei, form functional synapses through their recurrent axon collaterals, establishing a feedback control over striatal neuron activity (Tunstall et al., 2002; Guzman et al., 2003; Koos et al., 2004; Gustafson et al., 2006). Inputs from parvalbumin-positive GABAergic interneurons are another important source of synaptic inhibition of projection neurons, giving rise to a feedforward inhibitory pathway that is independent of striatal output (Plenz, 2003; Gustafson et al., 2006; Tepper et al., 2007). Here, we have shown that BC1 RNA is expressed on both parvalbumin-positive and parvalbumin-negative striatal GABA cells, implying that both feedback and feedforward inhibitory pathways are under the control of this small RNA in the striatum.

It has been hypothesized that BC1 RNA may be a general, nonspecific repressor of translation (Wang et al., 2002). This does not seem to be its physiological role, because at least the D₂DR mRNA translation is not regulated by BC1 RNA. We demonstrated that BC1 RNA can coregulate, together with FMRP, translation of specific mRNAs (Zalfa et al., 2003). Among these mRNAs is the one encoding MAP1B, raising the interesting possibility that BC1 RNA may affect somehow microtubule composition and hence axonal/dendritic transport. This could arguably lead to alterations in the efficiency of receptor insertion and

thus of their activity and stability. Additional investigations are required to address these possibilities.

Striatal dysfunction might contribute to explain the reduced exploration and increased anxiety observed in BC1-KO mice (Lewejohann et al., 2004), because both behavioral aspects involve striatal neuron network (Sareen et al., 2007). In particular, the increased inhibition of GABA transmission in BC1-KO mice could directly explain their increased anxiety, as reported previously for other mouse models (Kash et al., 1999).

Despite the fact that D₂ and CB₁ receptors control inhibitory synaptic transmission through similar molecular mechanisms (Centonze et al., 2005), ablation of BC1 gene did not result in widespread dysregulation of synaptic transmission, because the sensitivity of cannabinoid CB₁ receptors was intact in the striatum of BC1-KO mice. Furthermore, we also failed to observe changes of D₂DR sensitivity in mice lacking the FMR1 gene, indicating that FMRP–BC1 RNA interaction was not involved in the rearrangement of DA system observed in BC1-KO mice, suggesting

that different BC1 mRNPs have independent roles in neurons and/or brain areas.

Inhibition of GABA transmission is a prominent effect of D₂DR stimulation in the striatum, and both D₂L and the D₂S isoforms of these receptors are implicated in this action (Centonze et al., 2004). D₂L and D₂S receptors differ by the presence of an additional 29 amino acids within the third intracellular loop of the D₂L receptor (Usiello et al., 2000). Using primers able to amplify both isoforms, we detected no difference between WT and BC1-KO mice for both D₂L and D₂S.

In conclusion, in the present study, we identified BC1 RNA as a regulator of DA transmission in the striatum. The BC1-dependent modulation of D₂ receptors might be involved in the modifications of the efficacy of D₂DR transmission seen in physiological and pathological conditions, implying that dysregulation of BC1 RNA expression could mediate pathological alterations of DA transmission.

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