

Acknowledgements

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Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5

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Cocaine enhances dopamine-mediated neurotransmission by blocking dopamine re-uptake at axon terminals. Most dopamine-containing nerve terminals innervate medium spiny neurons in the striatum of the brain. Cocaine addiction is thought to stem, in part, from neural adaptations that act to maintain equilibrium by countering the effects of repeated drug administration^{1,2}. Chronic exposure to cocaine upregulates several transcription factors that alter gene expression and which could mediate such compensatory neural and behavioural changes^{1–4}. One such transcription factor is Δ FosB, a protein that persists in striatum long after the end of cocaine exposure^{3,5}. Here we identify *cyclin-dependent kinase 5* (*Cdk5*) as a downstream target gene of Δ FosB by use of DNA array analysis of striatal material from inducible transgenic mice. Overexpression of Δ FosB, or chronic cocaine administration, raised levels of *Cdk5* messenger RNA, protein, and activity in the striatum. Moreover, injection of *Cdk5* inhibitors into the striatum potentiated behavioural effects of repeated cocaine administration. Our results suggest that changes in *Cdk5* levels mediated by Δ FosB, and resulting alterations in signalling involving D1 dopamine receptors, contribute to adaptive changes in the brain related to cocaine addiction.

Transgenic mice displaying inducible and targeted expression of Δ FosB in the nucleus accumbens and caudatoputamen—together, the striatum of the brain—were engineered⁶. Analysis of complementary DNA expression array profiles from mice overexpressing Δ FosB indicated that the neuronal protein kinase *Cdk5* was a downstream target gene for Δ FosB in these brain regions (Fig. 1a). This effect was confirmed by quantitative *in situ* hybridization analyses of coronal brain sections from mice that did (H₂O) or did not (Dox) overexpress Δ FosB (Fig. 1b, left). Increased expres-

sion of *Cdk5* mRNA in response to Δ FosB accumulation was evident in both the caudatoputamen ($160.0 \pm 14.2\%$ of control, $P < 0.05$) and the nucleus accumbens ($152.6 \pm 12.9\%$, $P < 0.05$). Adult rats injected with cocaine for 8 days showed elevated levels of *Cdk5* mRNA in the caudatoputamen ($151.8 \pm 11.8\%$, $P < 0.05$) and nucleus accumbens ($150.5 \pm 10.0\%$, $P < 0.05$) in comparison to animals injected with saline (Fig. 1b, left). An increase was also observed in the level of mRNA encoding the neuron-specific *Cdk5*-activating cofactor, p35, in response to Δ FosB induction or chronic cocaine administration in both the caudatoputamen ($121.8 \pm 5.7\%$ and $129.8 \pm 7.5\%$, respectively, $n = 6$, $P < 0.05$) and the nucleus accumbens ($119.8 \pm 5.2\%$ and $126.3 \pm 7.4\%$, respectively, $n = 6$, $P < 0.05$) (Fig. 1b, right). Increased levels of *Cdk5* protein were observed in striatal tissue dissected from transgenic Δ FosB-expressing mice ($130 \pm 10\%$, $P < 0.05$) and from rats exposed chronically to cocaine ($180 \pm 20\%$, $P < 0.05$) compared to control animals (Fig. 1c, left and middle panels). Increased striatal p35 protein levels also occurred in response to chronic cocaine ($147.3 \pm 11\%$, $n = 12$, $P < 0.05$) (Fig. 1c, right panel). These results indicated that *Cdk5*, together with its activating cofactor p35, are downstream targets of chronic cocaine exposure, and raised the possibility that this protein kinase is involved in the behavioural effects of cocaine.

A characteristic behavioural effect of cocaine is potentiation of locomotor activity. The effects of daily intra-accumbens infusions of a potent *Cdk5* inhibitor, roscovitine, on cocaine-induced locomotor activity were examined over a 60-min period daily for 5 days. Administration of cocaine resulted in a marked and progressive increase in locomotor activity over this 5-day period, indicating the development of locomotor sensitization, during the initial 10–20 min period following injection (data not shown). Roscovitine infusions did not significantly affect locomotor responses to initial cocaine administration. However, roscovitine markedly potentiated the locomotor effects of repeated cocaine exposures. This was evident as an augmentation of cocaine's effects over successive days of injections (Fig. 2a). By day 4, significant differences were observed between the saline/cocaine and roscovitine/cocaine groups. By day 5, mean cocaine-induced activity rates for roscovitine-infused animals were almost double those measured for vehicle-infused animals. This effect of roscovitine was most evident 40–60 min after cocaine administration (Fig. 2b).

Repeated intra-accumbens infusions of a less selective *Cdk5* inhibitor, olomoucine, also potentiated cocaine's locomotor effects. This action was similar to that produced by roscovitine, except that a marked behavioural effect was already observed as early as day 3 (Fig. 2c). In response to cocaine treatment on days 4 and 5, olomoucine-treated animals exhibited stereotypy, which denotes enhanced sensitization and is known to compete with increases in locomotor activity¹⁰. In contrast, intra-accumbens infusions of the inactive congener, iso-olomoucine, failed to enhance either locomotor (Fig. 2c) or stereotypic responses to cocaine. These behavioural findings indicate that cocaine-induced increases in *Cdk5* levels may serve a homeostatic function to dampen responses to subsequent drug exposure.

One way in which *Cdk5* could regulate the psychomotor effects of chronic cocaine is through regulation of dopamine signalling. *Cdk5* phosphorylates a key molecule involved in striatal dopamine signalling, DARPP-32—dopamine and cyclic AMP-regulated phosphoprotein, M_r 32K—at threonine-75 (Thr75)⁷. Immunocytochemistry studies revealed that DARPP-32 and *Cdk5* are colocalized: all medium spiny neurons in the nucleus accumbens that contained DARPP-32 also contained *Cdk5* (Fig. 3a). Furthermore, dendrites in the neuropil were typically double-labelled and sometimes contained puncta that were immunoreactive to both proteins. Increased levels of phospho-Thr75 DARPP-32 were observed in striatal tissue dissected from transgenic mice overexpressing Δ FosB (Fig. 3b). In rats subjected to chronic cocaine

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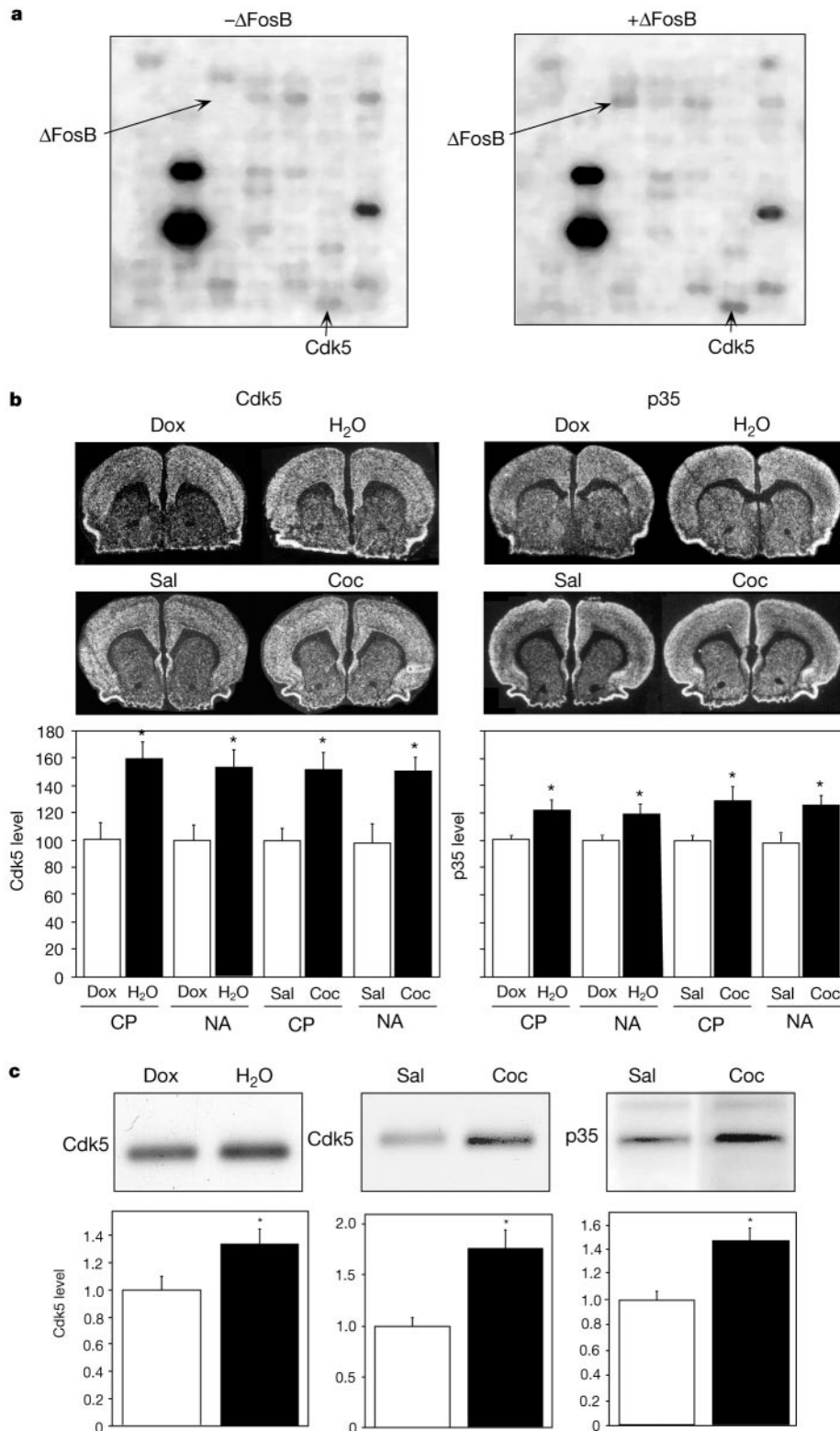


Figure 1 Increased expression of Cdk5 in inducible transgenic mice overexpressing Δ FosB and in rats chronically treated with cocaine. **a**, cDNA expression arrays probed with radiolabelled cDNA from control mice ($-\Delta$ FosB) (left) or transgenic mice overexpressing Δ FosB ($+\Delta$ FosB) (right). The positions of oligonucleotides encoding Δ FosB and Cdk5 are indicated. **b**, Comparison of the levels of Cdk5 (left) and p35 (right) gene expression by *in situ* hybridization. Representative labellings are shown for sections from inducible transgenic mice on (Dox) or off doxycycline (H₂O) (top panels) and rats treated chronically with saline (Sal) or cocaine (Coc; middle panels). Levels of signals in

the caudatoputamen (CP) and nucleus accumbens (NA) are shown in bar graphs. **c**, Comparison of the levels of Cdk5 and p35 protein. Representative Cdk5 and p35 immunoblots of striatal tissue dissected from inducible transgenic mice on or off doxycycline (left) and from rats treated chronically with saline or cocaine (middle, right) are shown with levels. Data represent means \pm s.e.m. for $n = 6$; asterisk indicates $P < 0.05$ compared to control, Student's *t*-test. Data for Cdk5 and p35 levels are expressed in arbitrary units.

administration, the levels of phospho-Thr 75 DARPP-32 were increased in both the caudatoputamen and nucleus accumbens (Fig. 3c, d). Total levels of DARPP-32 were unaffected under these conditions.

Cdk5-dependent phosphorylation of DARPP-32 at Thr 75 reduces the efficacy of dopamine/cyclic AMP/protein kinase A/DARPP-32/protein phosphatase-1 signalling⁷. Various aspects of this signalling pathway were examined. Treatment of striatal slices

from saline-injected rats with the D1 receptor agonist, SKF 81297, is known to increase PKA-dependent phosphorylation of DARPP-32 (ref. 8), ARPP-16 (ref. 9) and ARPP-21 (ref. 10). These effects of the D1 agonist were attenuated in striatal slices from rats exposed to chronic cocaine (Fig. 4a). Decreased PKA-dependent phosphorylation of DARPP-32 (at Thr 34) and of the GluR1 subunit of the AMPA-type glutamate receptor (at Ser 845) was observed in striatal tissue dissected from rats chronically treated with cocaine (Fig. 4b). Consistent with these effects being under the control of Cdk5, roscovitine treatment restored PKA-dependent phosphorylation of DARPP-32 at Thr 34 (10.6 ± 2.1 -fold increase, $n = 6$, $P = 0.0002$) and the GluR1 receptor at Ser 845 (5.2 ± 1.6 -fold increase, $n = 6$, $P = 0.006$) in striatal slices from chronic-cocaine-treated rats. Consistent with the ability of PKA-dependent phosphorylation of GluR1 to increase the conductance of AMPA/kainate channels¹¹, we found reduced peak amplitudes of AMPA/kainate-evoked currents in striatal neurons from chronic-cocaine-treated rats (Fig. 4c). These results expand upon the observation that nucleus accumbens neurons from rats treated with a psychomotor stimulant were less sensitive to the current-dependent increases in firing rates of striatal dopaminergic neurons caused by glutamate¹².

It has been suggested that chronic administration of cocaine, as well as other drugs of abuse, lead to enhancement of D1/PKA

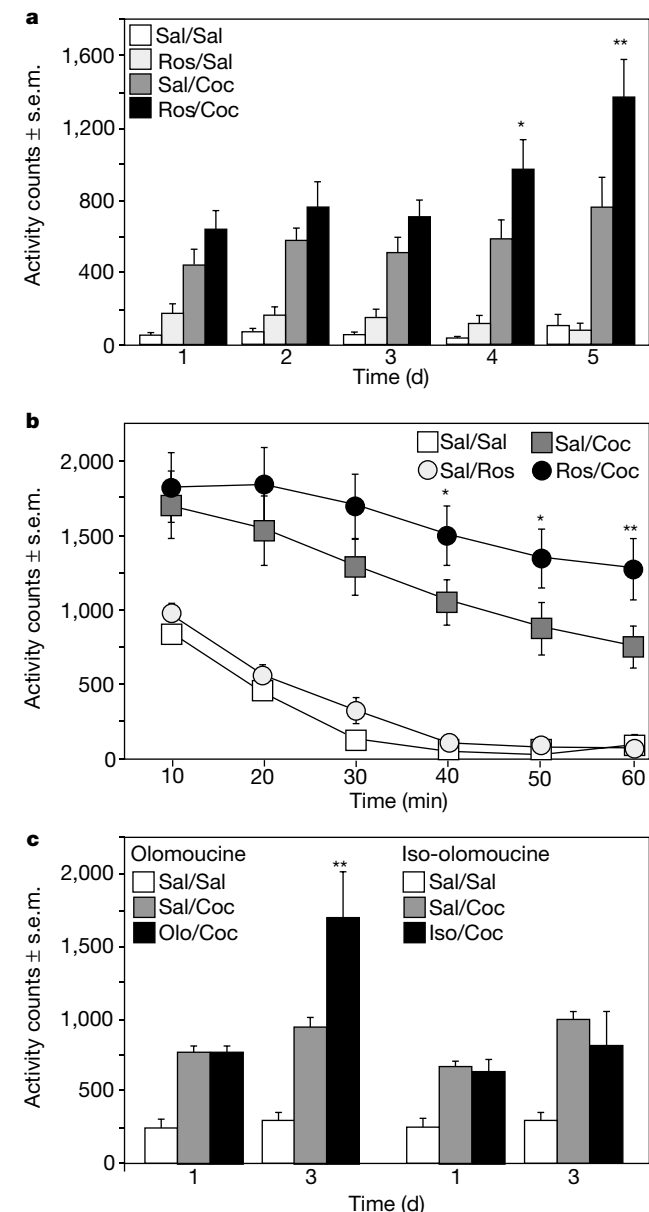


Figure 2 Effect of Cdk5 inhibitors on locomotor behavioural response to repeated cocaine injections. **a**, Locomotor activity for rats, given intra-accumbens infusions of saline (Sal) or roscovitine (Ros) and i.p. injections of saline (Sal) or cocaine (Coc), from 50 to 60 min post-injection on successive days 1–5. **b**, Behavioural effects of each treatment on day 5 measured for 60 min post-injection with values plotted at 10-min intervals. **c**, Behavioural effects of intra-accumbens infusion of olomoucine (Olo; left panel) or the inactive analogue, iso-olomoucine (Iso; right) on cocaine (i.p.)-induced locomotor activity from 40–50 min post-injection at days 1 and 3 of treatment. All data represent mean activity counts \pm s.e.m. measured by photocell over test period; asterisk indicates $P < 0.05$, double asterisk indicates $P < 0.001$ by analysis of variance and *post hoc* Scheffé's *F*-test. For day 3 in panel **a**, $P < 0.07$. For panels **a** and **b**, Sal/Sal, $n = 6$; Sal/Coc, $n = 11$; Ros/Sal, $n = 9$; Ros/Coc, $n = 11$. For panel **c**, Sal/Sal, $n = 6$, Sal/Coc, $n = 9$, Olo/Coc, $n = 11$; Iso/Coc, $n = 11$.

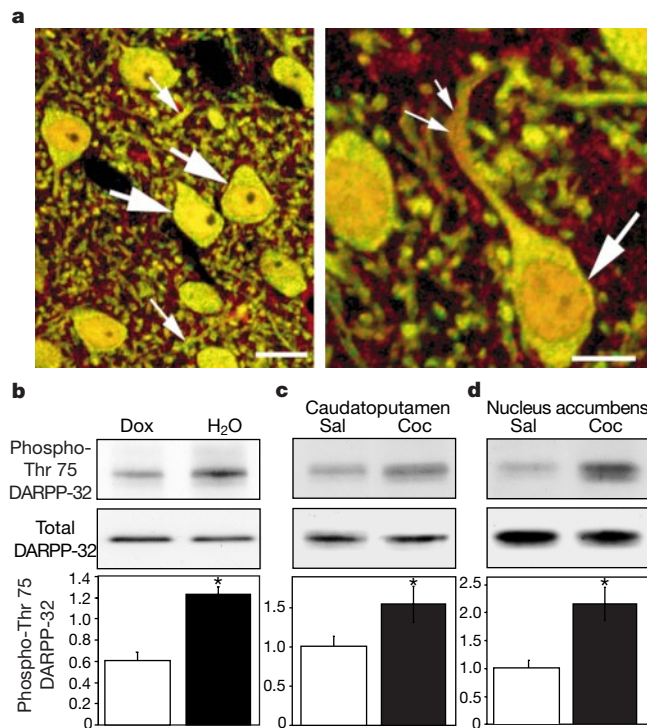


Figure 3 Increased phosphorylation of DARPP-32 by Cdk5 in inducible transgenic mice overexpressing Δ FosB and in rats chronically treated with cocaine. **a**, Laser confocal photomicrograph of tissue co-immunolabelled for Cdk5 (red) and DARPP-32 (green) in the nucleus accumbens. Overlapping signal appears prominently as yellow. All neurons that immunostained for DARPP-32 also immunostained for Cdk5 (see larger arrows for examples). Dendrites in the neuropil were also double-stained (small arrows). Images were acquired separately in each channel (dual scan mode) to eliminate the possibility of signal bleed-over from one channel to the other. Scale bar, 10 μ m. **b**, Quantitative immunoblot analysis of level of phospho-Thr 75 DARPP-32 in striatal tissue dissected from inducible Δ FosB transgenic mice on or off doxycycline. Representative blots are shown for phospho-Thr 75 DARPP-32 and total DARPP-32 in the upper two panels and levels are shown in the lower panel. **c**, **d**, Level of phospho-Thr 75 DARPP-32 in caudatoputamen (**c**) and nucleus accumbens (**d**) from rats treated with saline or chronic cocaine for 10 d. Data represent means \pm s.e.m. for $n = 6$; asterisk indicates $P < 0.05$, Mann-Whitney non-parametric *t*-test.

signalling^{2,13}. However, our results indicate that at least some target substrates exhibit reduced D1/PKA-dependent phosphorylation following chronic exposure to cocaine. Our observations of increased Cdk5 in response to cocaine, which can cause an increase in phospho-Thr 75 DARPP-32 and a reduction in PKA activity, provides a mechanism for differential regulation of PKA-dependent phosphorylation by chronic cocaine. It is possible that the Δ FosB/Cdk5/phospho-Thr 75 DARPP-32 pathway is targeted towards particular substrates or subcellular elements and away from others. Moreover, the effects of chronic cocaine and other drugs of abuse on dopamine signalling may be subject to variations in dosing parameters, period of withdrawal, and paradigms of study.

There is now considerable evidence that the D1 receptor is important in mediating the behavioural effects of cocaine^{12,14–16}. We have reported that an acute dose of cocaine activates PKA, causing an increase in phosphorylation of DARPP-32 at Thr 34 while causing a decrease in phosphorylation at Thr 75 via a signalling mechanism that depends on protein phosphatase 2A¹⁷. The data

reported here support a distinct biochemical scheme in which repeated exposure to cocaine causes accumulation of Δ FosB, which in turn results in increased expression of Cdk5; increased Cdk5-dependent phosphorylation of DARPP-32 at Thr 75 then attenuates D1/PKA signalling. In support of the behavioural relevance of this scheme, mice lacking the *DARPP-32* gene¹⁸ and rats treated with Cdk5 inhibitors (Fig. 2) both exhibit enhanced behavioural responses to chronic administration of cocaine.

Previous studies established that induction of Δ FosB expression in the striatum enhances behavioural responsiveness to cocaine⁴. Thus Δ FosB-mediated induction of Cdk5 would appear to oppose the overall effect of this transcription factor, suggesting that Δ FosB—which acts on numerous genes—mediates both sensitizing and compensatory adaptations. The ability of intra-accumbens infusions of Cdk5 inhibitors to potentiate the locomotor effects of repeated cocaine administration suggests that the Cdk5/DARPP-32 pathway plays a negative feedback homeostatic role with respect to the behavioural effects of cocaine. It remains to be determined whether other actions of Cdk5^{19–21} contribute to the behavioural effects of cocaine. Nevertheless, by combining DNA array analyses, an inducible transgenic animal model, and behavioural studies, we have now identified Cdk5 as a target that alters dopamine signal transduction as part of the long-term plasticity associated with cocaine addiction. □

Methods

For cDNA expression arrays, total RNA was isolated with the RNAqueous phenol-free total RNA isolation kit (Ambion) from dissected striatal tissue from either control mice carrying only the *NSE-tTA* gene ($-\Delta$ FosB) or Δ FosB-inducible transgenic mice carrying both *Tetop- Δ FosB* and *NSE-tTA* genes, which had been fed 100 mg l⁻¹ doxycycline to inhibit transgene expression, followed by 12 weeks in the absence of doxycycline ($+\Delta$ FosB). Poly(A)⁺ RNA was isolated from total RNA using an Oligotex mRNA isolation kit (Qiagen), and used as template for synthesis of ³²P-labelled cDNA probes. The probes were hybridized to Atlas cDNA expression arrays containing 588 genes (Clontech) according to the manufacturer's suggestions.

Δ FosB-inducible transgenic mice were either fed 100 mg l⁻¹ doxycycline or denied doxycycline for 12 weeks. Adult male Sprague-Dawley rats initially weighing between 160 and 240 g were injected with cocaine or vehicle (intraperitoneal, i.p.; 20 mg kg⁻¹, 0.9% NaCl) at the same time each day for 8 d. All analyses were initiated 12 h after the final dose. For *in situ* hybridization studies, [α -³⁵S]UTP-labelled riboprobes were prepared by *in vitro* transcription from cDNA clones corresponding to full-length clones of rat Cdk5 and p35²². Cryostat sections were prepared and hybridized as previously described²³. After hybridization, the sections were exposed to Biomax MR film (Kodak) for 2–6 d and analysed with a Microcomputer Imaging Device system (M4, Imaging Research, Inc.). Statistical analyses of the data were performed using the two-tailed unpaired Student's *t*-test. Striatal tissue was rapidly dissected on ice and homogenized in boiling lysis buffer containing 1% SDS and 50 mM sodium fluoride with sonication. Striatal slice preparation and treatment were conducted as previously described⁷. Use of antibodies to phospho-Thr 34 DARPP-32², phospho-Thr 75 DARPP-32⁷, phospho-Ser 55 ARPP-21¹⁰, phospho-Ser 88 ARPP-16^{7,9} and phospho-Ser 845 GluR1²⁴ for immunoblotting has been previously described.

Immunocytochemistry was performed as previously described²⁵ except that 0.3% glutaraldehyde was added to the fixative solution. Vibratome sections were incubated with a mixture of antibodies against DARPP-32 and Cdk5 (C-8, Santa Cruz Biotechnologies, Inc.) overnight at 25 °C. After a 30-min rinse in buffer, sections were incubated in a mixture of fluorescein-isothiocyanate-labelled goat antimouse IgG and Cy3-labelled goat anti-rabbit IgG for 1 h, followed by three 30-min washes in buffer. Sections were mounted in Vectashield and analysed with a Zeiss 410 confocal laser scanning microscope. Images were acquired separately in each channel (dual scan mode) to eliminate the possibility of signal bleed-over from one channel to the other.

Drug-induced alterations in cocaine sensitization were measured in adult male Sprague-Dawley rats according to published procedures for surgery, drug infusion, apparatus and behavioural methods^{26,27}. Coordinates for the nucleus accumbens were anterior-posterior 1.7 from bregma, medial-lateral \pm 1.5 from midline, dorso-ventral -6.0 from skull. Cocaine hydrochloride was administered at 15 mg kg⁻¹ i.p. in sterile 0.9% sodium chloride. Roscovitine, olomoucine and iso-olomoucine were dissolved in sterile phosphate-buffered saline:DMSO (50:50) and 40 nmol were microinfused in 0.5 μ l over a 2-min period (compare ref. 28). One week after surgery and 2 d after habituation to the locomotor chambers, subjects received five daily injections of cocaine (15 mg kg⁻¹, i.p.) or saline 20 min after bilateral intra-accumbens vehicle, roscovitine, olomoucine or iso-olomoucine infusions. Drugs were given at the same time each day. Subjects were then placed into the chambers and activity was monitored for 60 min.

For electrophysiological studies, acutely dissociated striatal neurons were prepared as previously described²⁹. Whole-cell recordings of voltage-gated AMPA/kainate-activated current (100 μ M kainate) were measured using standard whole-cell voltage-clamp techniques³⁰. Summary data are presented in box plot format.

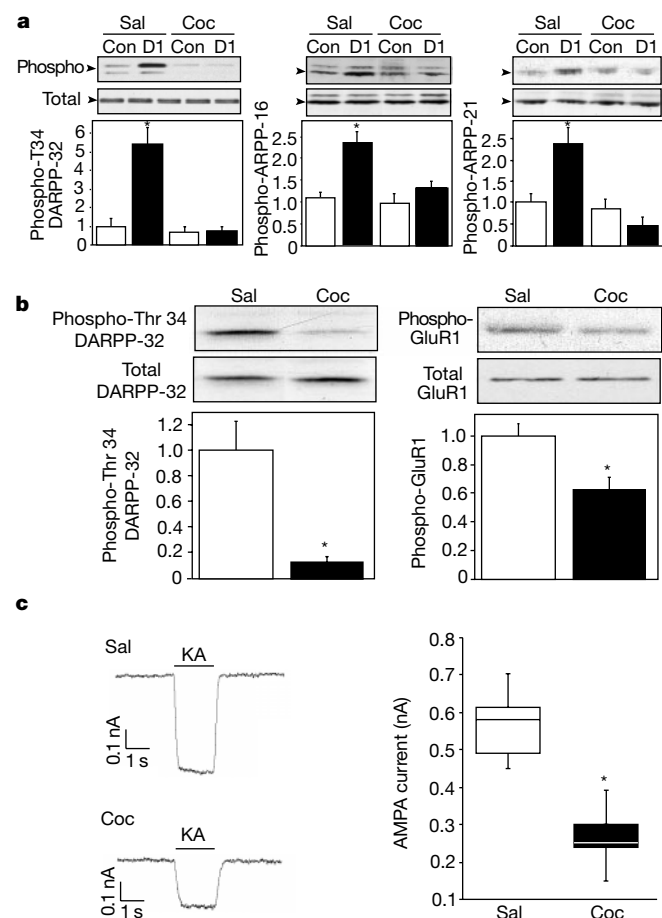


Figure 4 Effects of chronic cocaine exposure on dopamine/PKA signalling in the striatum. **a**, Effect of the selective D1 agonist SKF 81297 (D1), in comparison to untreated controls (Con), on PKA phosphorylation of DARPP-32 (left), ARPP-21 (middle) and ARPP-16 (right) in striatal slices from saline- and cocaine-treated rats. Representative blots are shown for proteins phosphorylated by PKA (top panels) and total proteins (middle panels). Levels are shown in the bottom panels. **b**, Effects of chronic cocaine exposure on PKA phosphorylation of DARPP-32 and GluR1 in tissue *in vivo*. Data in **a** and **b** represent means \pm s.e.m., asterisk indicates $P < 0.01$ versus controls, Mann-Whitney non-parametric *t*-test, $n = 6$. **c**, Effect of chronic cocaine on ligand-gated AMPA/kainate current. Kainate (KA)-sensitive AMPA current recordings in saline- or cocaine-treated rats (left) and statistical analyses (right) are shown. Data represent means \pm s.e.m., asterisk indicates $P < 0.01$ versus controls, unpaired *t*-test, $n = 6$.

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BRI1 is a critical component of a plasma-membrane receptor for plant steroids

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Most multicellular organisms use steroids as signalling molecules for physiological and developmental regulation. Two different modes of steroid action have been described in animal systems: the well-studied gene regulation response mediated by nuclear receptors^{1,2}, and the rapid non-genomic responses mediated by proposed membrane-bound receptors^{3,4}. Plant genomes do not seem to encode members of the nuclear receptor superfamily⁵. However, a transmembrane receptor kinase, brassinosteroid-insensitive1 (BRI1), has been implicated in brassinosteroid responses^{6,7}. Here we show that BRI1 functions as a receptor of brassinolide, the most active brassinosteroid. The number of brassinolide-binding sites and the degree of response to brassinolide depend on the level of BRI1 protein. The brassinolide-binding activity co-immunoprecipitates with BRI1, and requires a functional BRI1 extracellular domain. Moreover, treatment of *Arabidopsis* seedlings with brassinolide induces autophosphorylation of BRI1, which, together with our binding studies, shows that BRI1 is a receptor kinase that transduces steroid signals across the plasma membrane.

Brassinosteroids (BRs) are involved in a wide range of plant developmental processes⁸. Mutant plants deficient in BR biosynthesis, such as *det2*, show phenotypes of dwarfism, delayed senescence, reduced fertility, and light-independent development^{9,10}. Mutations in the *BRI1* gene cause BR-insensitivity and morphological phenotypes nearly identical to BR biosynthetic mutants, suggesting an important and specific role for *BRI1* in BR perception or signal transduction^{11,12}. The *BRI1* gene encodes a receptor kinase that has an extracellular domain containing 25 leucine-rich repeats (LRRs), which are interrupted by a 70-amino-acid island, a transmembrane domain, and a cytoplasmic kinase domain with serine/threonine specificity^{6,13}. The structure of BRI1 and its plasma membrane localization¹³ support the hypothesis that BRI1 interacts with an extracellular ligand, which is either BR itself or a secondary signal generated by BR perception, and the signal is transduced through the kinase. Consistent with this hypothesis, recent studies using a chimaeric receptor approach showed that the extracellular domain of BRI1 could confer brassinolide (BL) responsiveness to the intracellular kinase domain of a heterologous LRR kinase⁷.

To test whether BL is the ligand that directly activates the BRI1 receptor kinase, we first analysed the effect of overexpression of BRI1 on BL binding activity in membrane fractions. Transgenic *Arabidopsis* plants overexpressing a BRI1-GFP fusion protein¹³ (GFP, green fluorescent protein) showed reduced inhibition of hypocotyl growth by a BR biosynthesis inhibitor¹⁴ (Fig. 1a, b). They also had longer petioles, similar to plants overexpressing the BR biosynthetic enzyme DWF4¹⁵ (Z.W. and J.C., unpublished data) (Fig. 1c). These phenotypes are consistent with the interpretation that overexpression of the BRI1-GFP protein increases the response of *Arabidopsis* to BRs. We observed a dramatic increase of BL binding activity in the membrane fractions of the BRI1-GFP transgenic plants (Fig. 1d). The increase of binding was due to an