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CIN85 regulates dopamine receptor endocytosis and governs behaviour in mice

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Despite extensive investigations of Cbl-interacting protein of 85 kDa (CIN85) in receptor trafficking and cytoskeletal dynamics, little is known about its functions *in vivo*. Here, we report the study of a mouse deficient of the two CIN85 isoforms expressed in the central nervous system, exposing a function of CIN85 in dopamine receptor endocytosis. Mice lacking CIN85 exon 2 (CIN85$^{\Delta ex2}$) show hyperactivity phenotypes, characterized by increased physical activity and exploratory behaviour. Interestingly, CIN85$^{\Delta ex2}$ animals display abnormally high levels of dopamine and D2 dopamine receptors (D2DRs) in the striatum, an important centre for the coordination of animal behaviour. Importantly, CIN85 localizes to the post-synaptic compartment of striatal neurons in which it co-clusters with D2DRs. Moreover, it interacts with endocytic regulators such as dynamin and endophilins in the striatum. Absence of striatal CIN85 causes insufficient complex formation of endophilins with D2DRs in the striatum and ultimately decreased D2DR endocytosis in striatal neurons in response to dopamine stimulation. These findings indicate an important function of CIN85 in the regulation of dopamine receptor functions and provide a molecular explanation for the hyperactive behaviour of CIN85$^{\Delta ex2}$ mice.

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Introduction

Cbl-interacting protein of 85 kDa (CIN85)/SH3-domain kinase binding protein 1 (SH3KBP1) forms, together with CMS (p130Cas ligand with multiple SH3 domains)/CD2-associated protein (CD2AP), a family of adaptor molecules with established functions in coordinating the spatial and temporal assembly of protein complexes during receptor endocytosis, formation of kidney glomeruli and organization of the immunological synapse in T cells (Dikic, 2002). Common to both CIN85/SH3KBP1 (alias CIN85) and CMS/CD2AP is a core structural organization comprising three N-terminal SH3 domains, followed by a centrally located proline-rich region and a C-terminal coiled coil (Dikic, 2002). In addition, in a functional perspective, both family members share certain characteristics, given that their SH3 domains specifically recognize a consensus Px(P/A)xxR motif, present in Cbl (Petrelli *et al*, 2002; Soubeyran *et al*, 2002; Kurakin *et al*, 2003; Kobayashi *et al*, 2004; Jozic *et al*, 2005; Moncalian *et al*, 2006) and other proteins involved in endocytosis, including synaptojanin, Huntingtin-interacting protein 1-related, Alix and Dab2 (Chen *et al*, 2000; Kowanetz *et al*, 2004), as well as regulators of the actin cytoskeleton, such as cortactin and the actin-capping protein CAPZ (Kirsch *et al*, 2001; Welsch *et al*, 2001; Cormont *et al*, 2003; Hutchings *et al*, 2003; Lynch *et al*, 2003). Indeed, a function of CIN85 and CMS/CD2AP in the regulation of actin dynamics is emphasized by their ability to directly interact with F-actin and induce bundling of actin filaments (Gaidos *et al*, 2007). The multitude of verified interaction partners has placed CIN85

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as a central adaptor molecule involved in the recruitment of the endocytic machinery required for the internalization of a variety of cell surface receptors, including growth factor receptors (such as EGFR, Met and VEGFR) (Petrelli *et al*, 2002; Soubeyran *et al*, 2002; Kobayashi *et al*, 2004), immunoglobulin IgE receptors in mast cells (Molfetta *et al*, 2005), as well as during the infectious internalization of the bacterial pathogen *Listeria monocytogenes* (Veiga and Cossart, 2005).

Despite sharing many overlapping molecular functions in cultured cells, investigations in mice deficient of CD2AP have highlighted that CIN85 and CD2AP may have distinct functions *in vivo*. In agreement with its abundance in the kidney, mice lacking CD2AP suffer from a nephrotic syndrome caused by defective podocyte functionality during the formation of the glomerular slit diaphragm, eventually resulting in lethality at 6–8 weeks of age (Shih *et al*, 1999). An important function for CD2AP in spermatocyte production has furthermore been established, given that podocyte-specific reintroduction of transgenic CD2AP in knockout mice is sufficient to rescue animals into adulthood, but renders male mice infertile (Grunkemeyer *et al*, 2005). Interestingly, the podocyte and the cells of the basal seminiferous tubule are both cell types in which CIN85 is poorly expressed.

As a result of alternative splicing and differential promoter usage, the mouse CIN85 genomic locus gives rise to multiple isoforms, including CIN85-xl, CIN85-l (CIN85), CIN85-ΔA, CIN85-m, CIN85-s, CIN85-t and CIN85-h (Supplementary Figure S1) (Buchman *et al*, 2002; Finniss *et al*, 2004). The different isoforms display specific patterns of expression, among which CIN85-xl and CIN85-l are most abundant in brain and CIN85-l and CIN85-ΔA in thymus and spleen (Figure 2D; Supplementary Figure S1; Buchman *et al*, 2002). Lower levels of various isoforms can also be detected in kidney, muscle, skin, heart, lung and testis (Buchman *et al*, 2002 and unpublished observations). CD2AP is on the other hand predominantly expressed in spleen, thymus, heart, kidney, lung, muscle and liver, but seems to be lacking in neuronal cells (Dustin *et al*, 1998; Shih *et al*, 1999; Li *et al*, 2000).

In this study, we report a novel function of CIN85 in the regulation of post-synaptic dopamine receptor endocytosis in striatal neurons. In wild-type mice, CIN85 resides post-synaptically and associates with endocytic regulators, such as dynamin and endophilins. In mouse striatal neurons, absence of brain-specific CIN85 expression results in inefficient complex formation between dopamine receptors and endophilins, as well as reduced internalization of stimulated dopamine receptors. Mice deficient of brain-specific CIN85 expression show hyperactive phenotypes, which in many ways resemble the behavioural aberrations displayed in human beings affected by attention deficit hyperactivity disorder (ADHD), a disorder strongly associated with abnormal dopamine signalling.

Results

Specific isoforms of CIN85 localize post-synaptically in neurons

As CIN85 is highly expressed in the brain (Buchman *et al*, 2002), we were interested to investigate the function of CIN85 in the central nervous system (CNS). We initially analysed the expression pattern of CIN85 in various brain regions prepared from both mouse and rat (Figure 1A; Supplementary Figure S2 and data not shown) and found both of the major isoforms expressed in brain, CIN85-xl and CIN85-l, to be abundant in most brain regions tested, in line with earlier reported observations (Bian *et al*, 2008). To refine our survey of CIN85 localization on a sub-cellular level, we subsequently performed immunohistochemical analysis of endogenous CIN85 in cultured primary rat hippocampal neurons with mature synapses. In agreement with earlier reported observations (Kawata *et al*, 2006), and as shown in Figure 1B, we found high levels of CIN85 in the somatodendritic compartment, in which it frequently clustered in dendritic shafts, as well as within dendritic spines. Dendritic spines are small protrusions extending from the surface of dendrites, which are believed to be the main sites of excitatory synapses and thus vital centres for synaptic transmission in the brain (Segal, 2005).

We confirmed the localization of CIN85 in dendritic spines by showing a co-localization of CIN85 with F-actin, which is abundant in these structures (Figure 1B, upper panel). In addition, by counter-staining primary hippocampal neurons for CIN85 together with markers for pre-synaptic (synaptophysin) and post-synaptic (PSD-95) compartments, we found CIN85 to co-localize with PSD-95 at post-synaptic sites, closely juxtaposing synaptophysin (Figure 1B, middle and lower panel, respectively). In addition, in cultured primary striatal neurons, CIN85 was enriched in spine-like structures in dendrites (Figure 1C). Aiming at further characterizing the localization of CIN85 in neurons, we subsequently isolated synaptosomes, which are enriched in pre- and post-synaptic structures (Booth and Clark, 1978), from whole mouse brain preparations, as well as from isolated mouse striata, using conventional Percoll step-gradient or sucrose density-gradient centrifugations, respectively. Whereas CIN85, PSD-95 and synaptophysin were all present in crude synaptosomal isolates from whole mouse brains, further extraction with Triton X-100, which is known to specifically solubilize pre-synaptic compartments and thus enrich post-synaptic components, depicted an enrichment of CIN85 together with PSD-95 in the post-synaptic fractions (Figure 1D). Similar results were obtained in isolated mouse striata in which CIN85 co-fractionated with PSD-95 in post-synaptic density preparations (Figure 1E).

Mice lacking brain-specific CIN85 isoforms are hyperactive

To investigate the function of CIN85 in the CNS, we next set out to generate mice deficient of the two major CIN85 isoforms expressed in the brain (CIN85-xl and CIN85-l). Using homologous recombination, we targeted exon 2 of the CIN85 genomic locus for deletion (Figure 2A) and could by a combinatorial approach based on Southern blot (Figure 2B) and PCR (Figure 2C; Supplementary Figure S3) confirm a successful integration of the targeting vector and absence of CIN85 exon 2 ($CIN85^{\Delta ex2}$). In agreement with the observed loss of $CIN85^{\Delta ex2}$ on the DNA level, we could furthermore confirm the absence of CIN85-xl and CIN85-l isoforms in brain, as well as CIN85-l in thymus and spleen, in $CIN85^{\Delta ex2}$ knockout mice by western blot analysis (Figure 2D). As expected, whereas all CIN85 protein variants encoded by transcripts initiated from promoter #1 (CIN85-xl, CIN85-l and the shorter CIN85-ΔCP) were abolished in $CIN85^{\Delta ex2}$

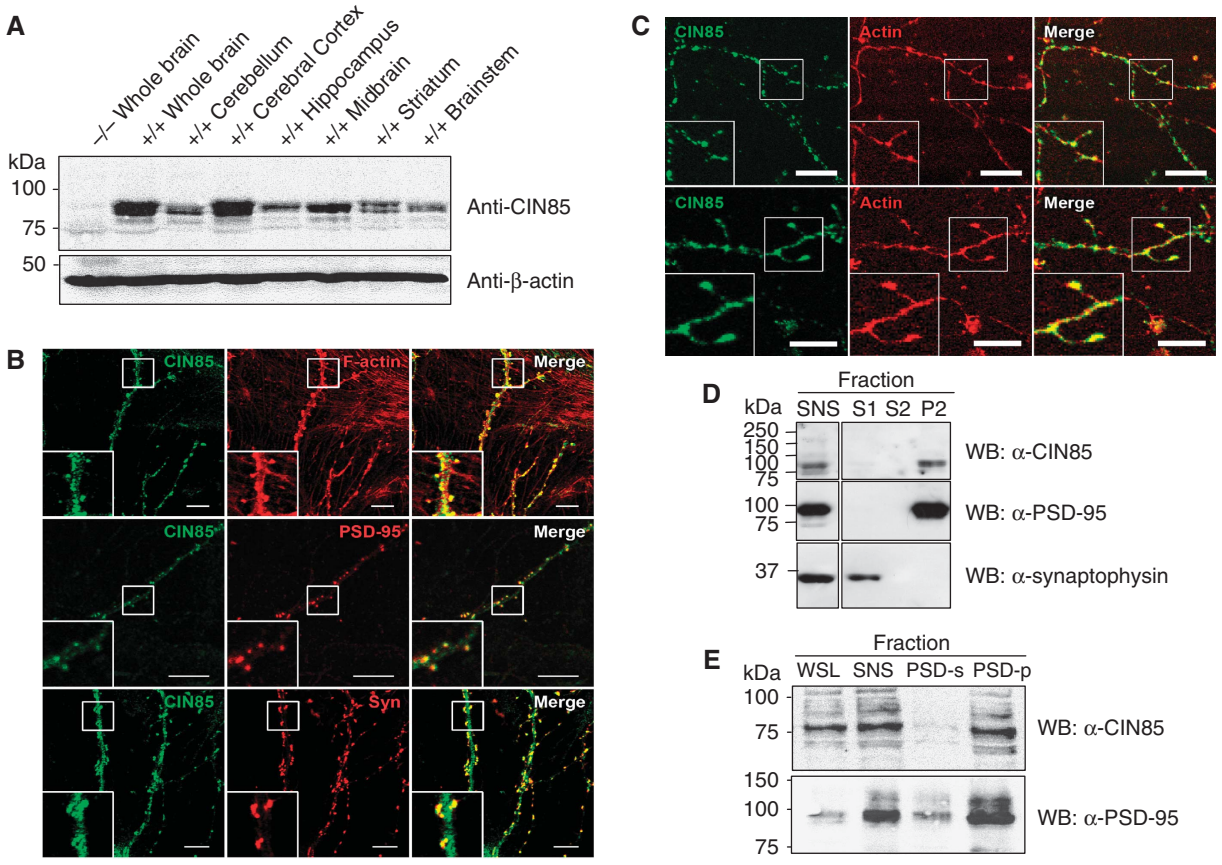


Figure 1 CIN85 is highly expressed in neurons in which it localizes to post-synaptic sites. (**A**) Western blot showing CIN85 expression in different mouse brain regions. Lysates (15 μg protein/lane) of the indicated brain regions from wild-type (+/+) and CIN85$^{\Delta ex2}$ knockout (−/−) mice were separated by 7% SDS–PAGE and immunoblotted with antibodies against CIN85 (CT). Equal protein loading was confirmed by re-blotting with anti-β-actin antibodies. (**B**) In primary hippocampal neurons derived from wild-type rat, CIN85 (SETA antibody, green) is localized to dendritic spines, in which it is accumulated in post-synaptic compartments, co-localizing with F-actin (red, upper panel) and PSD-95 (red, middle panel), closely juxtaposing the pre-synaptic synaptophysin protein (red, lower panel). Scale bars: 10 μm. (**C**) CIN85 (green) localizes to actin-positive (red), spine-like structures in dendrites of primary rat striatal neurons. Cells were fixed and stained with antibodies against CIN85 (SETA antibody, green) and with rhodamine phalloidin (red). Scale bars: 10 μm. (**D**) CIN85 is present in post-synaptic compartments in mouse brains. Synaptosome (SNS) fractions were isolated using the Percoll-step-gradient method from whole brain lysates as described in the Supplementary data. The western blot shows that CIN85, PSD-95 and synaptophysin are present in SNS fractions and that extraction with Triton X-100 solubilizes pre-synaptic synaptophysin into supernatant 1 (S1), whereas CIN85 and PSD-95 are present in post-synaptic fractions (pellet 2, P2, after a second Triton X-100 extraction). Supernatant 2 (S2) is the supernatant after the second Triton X-100 extraction. (**E**) CIN85 localizes to post-synaptic compartments in the striatum. Synaptosomes from mouse striata were prepared using a sucrose density-gradient method as described in the Supplementary data and immunoblotted with antibodies against CIN85 and PSD-95. WSL, whole striatal lysates; SNS, synaptosomes; PSD-s, supernatant of PSD fraction; PSD-p, pellet of PSD fraction. Each lane contains 15 μg of total protein.

mice, none of the transcripts using alternative, downstream promoters, such as that of CIN85-ΔA (Figure 2D; Supplementary Figure S1; Buchman *et al*, 2002), were affected by the induced recombination event. Importantly, the neo-cassette, which as a result of the homologous recombination event replaces exon 2 in CIN85$^{\Delta ex2}$ animals, has been designed with a terminal translational stop codon, which should make ribosomal read-through and re-initiation highly unlikely. Moreover, given that exons 1 and 3 are out of frame, we anticipate that splicing events skipping the introduced LacZ/neo-cassette should result in inappropriate and prematurely terminated translation.

Homozygous CIN85$^{\Delta ex2}$ mice are viable and fertile, and display no obvious abnormalities. However, as neurological phenotypes may not be evident at first glance and often progress with age, we subjected the CIN85$^{\Delta ex2}$ knockouts to extensive analyses of a broad range of parameters according to the physiological screens defined by the German Mouse Clinic, summarized in Supplementary Figure S4A and the Supplementary data. Among the parameters tested, the screen exposed a clear knockout-specific phenotype in energy metabolism, behaviour (see below) and gene expression profiles (data not shown). When comparing CIN85$^{\Delta ex2}$ knockout and wild-type animals, we found that mice deficient of CIN85-xl and CIN85-l showed deviations in several metabolic parameters, including increased energy uptake, higher lean mass and lower fat content (Figure 3A; Supplementary Figure S4B), characteristics that in many ways resemble the phenotypes earlier reported for c-Cbl knockout mice (Molero *et al*, 2004).

In agreement with a function of CIN85 in the CNS, CIN85$^{\Delta ex2}$ mice also displayed obvious aberrations in behaviour (Figure 3B). When subjected to the modified hole-board test, which assays spontaneous behaviour such as forward and vertical locomotor activity, speed of movement and exploratory behaviour in a novel environment (Supplementary Figure S4C), CIN85$^{\Delta ex2}$ mice showed significantly increased activities, as compared with wild-type

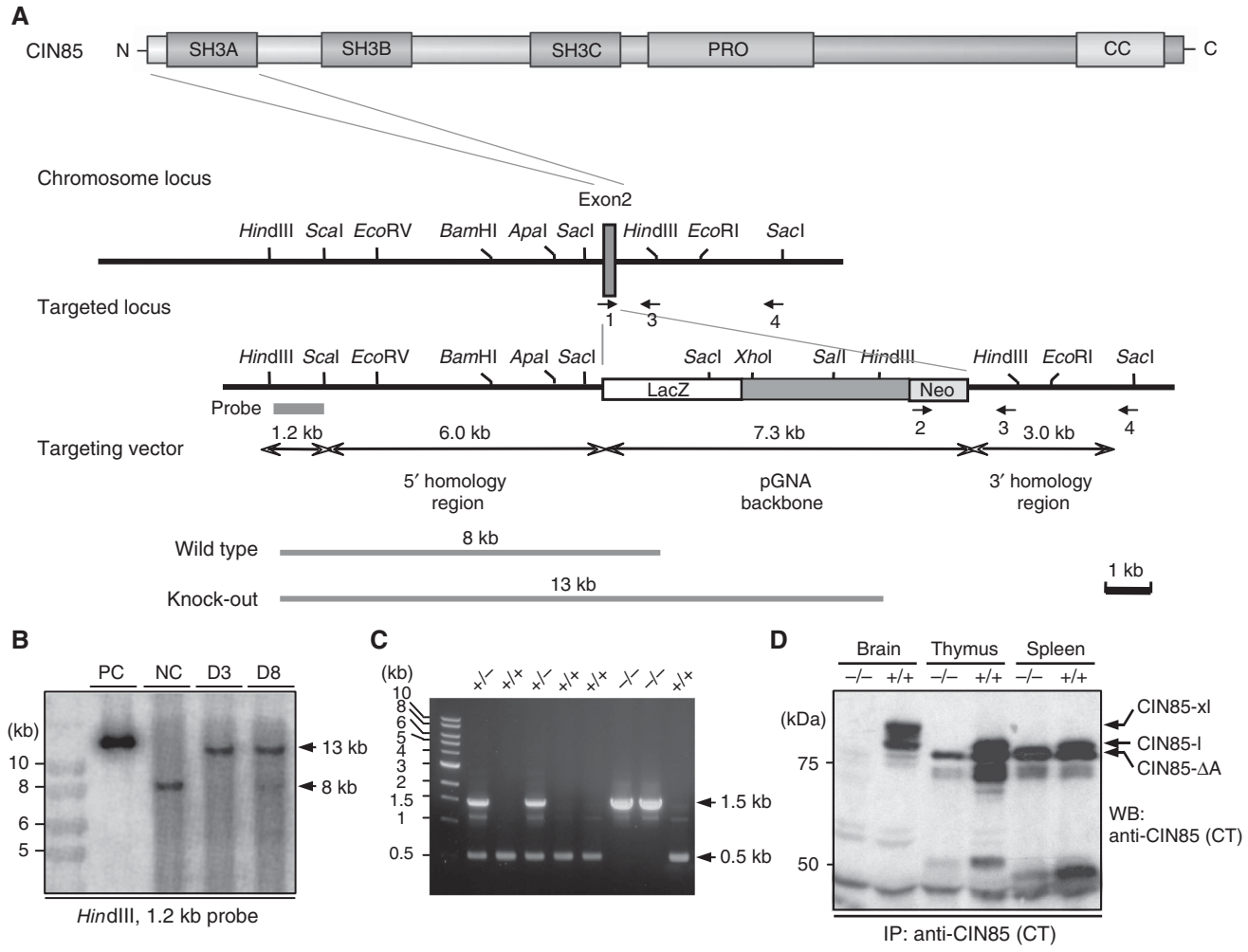


Figure 2 Generation of CIN85$^{\Delta ex2}$ knockout mice. (**A**) Gene targeting of the CIN85 locus by removal of exon 2 by homologous recombination. The targeting vector consisted of a 6.0 kb 5′ homology region, the pGNA backbone containing a LacZ/neomycin cassette followed by a 3.0 kb 3′ homology region. The wild-type and targeted loci with their respective restriction sites are indicated. The arrows (1, 2, 3, 4) represent the primers used for genotyping of ES cells and CIN85$^{\Delta ex2}$ knockout mice (see Supplementary Figure S3 for primer sequences). The 1.2 kb probe used for Southern blot detects an 8 kb product in wild-type mice and a 13 kb product in CIN85$^{\Delta ex2}$ knockout mice after *Hin*dIII digestion. (**B**) Confirmation of recombinant targeting events in ES cell clones by Southern blot. Genomic DNA from wild-type ES cells and targeted ES cell clones (D3 and D8) was digested with *Hin*dIII and analysed by Southern blotting using the probe indicated in (**A**). The two targeted ES cell clones showed a 13 kb mutant band, indicating successful integration of the targeting vector by homologous recombination, whereas the wild-type ES cells, as expected, showed the 8 kb band of the wild-type locus (NC). An extended form of a linearized targeting vector was used as a positive control (PC). (**C**) Confirmation of the targeting event in CIN85$^{\Delta ex2}$ knockout mice by PCR, using a mixture of primers 1, 2 and 3. CIN85$^{\Delta ex2}$ knockout mice (−/−) give rise to the expected 1.5 kb product (primers 2 and 3), wild-type mice (+/+) the expected 0.5 kb product (primers 1 and 3) and heterozygous mice (+/−) to both products (primers 1, 2 and 3). Primer sequences are found in Supplementary Figure S3A. (**D**) Whole tissue lysates from mouse brain, thymus and spleen (200 μg/lane) from wild-type (+/+) and CIN85$^{\Delta ex2}$ knockout mice (−/−) were subjected to immunoprecipitation with anti-CIN85 (CT) antibodies. Subsequent western blot analysis with antibodies against CIN85 confirms the removal of CIN85-xl and CIN85-l in the brain and of CIN85-l in thymus and spleen in CIN85$^{\Delta ex2}$ knockouts. CIN85-ΔA remains in thymus and spleen. Some smaller isoforms present in thymus and spleen are also removed in CIN85$^{\Delta ex2}$ knockouts.

littermates (Figure 3B). Specifically, CIN85$^{\Delta ex2}$ animals exhibited enhanced forward locomotor activity, manifested by an increase in total distance travelled, number of line crossings, mean and maximum velocity, as well as turning frequency (Figure 3B; Supplementary Figure S4C). In addition, CIN85$^{\Delta ex2}$ knockout mice showed enhanced exploratory behaviour—entering the board more frequently and exploring a larger number of holes on the board, than wild-type animals (Figure 3B). Increase in total time spent on the board just missed a significant genotype effect limit ($P = 0.06$, NS; Figure 3B). The increased physical activity displayed by the CIN85$^{\Delta ex2}$ animals should naturally be accompanied by an increased energy demand and indeed these mice show an increased energy intake (Figure 3A). However, given the lean body composition phenotype of these animals, the increased energy intake cannot completely compensate for the increased energy requirements. In summary, CIN85$^{\Delta ex2}$ mice are characterized by hyperactive behaviour, as evidenced by the increased locomotor and exploratory activities.

No obvious alterations in long-term potentiation, short-term synaptic plasticity or learning and memory in CIN85$^{\Delta ex2}$ knockout mice

The similarity of the metabolic phenotypes displayed by CIN85$^{\Delta ex2}$ and c-Cbl knockout mice is interesting in the light of a recent report indicating brain-specific activities of

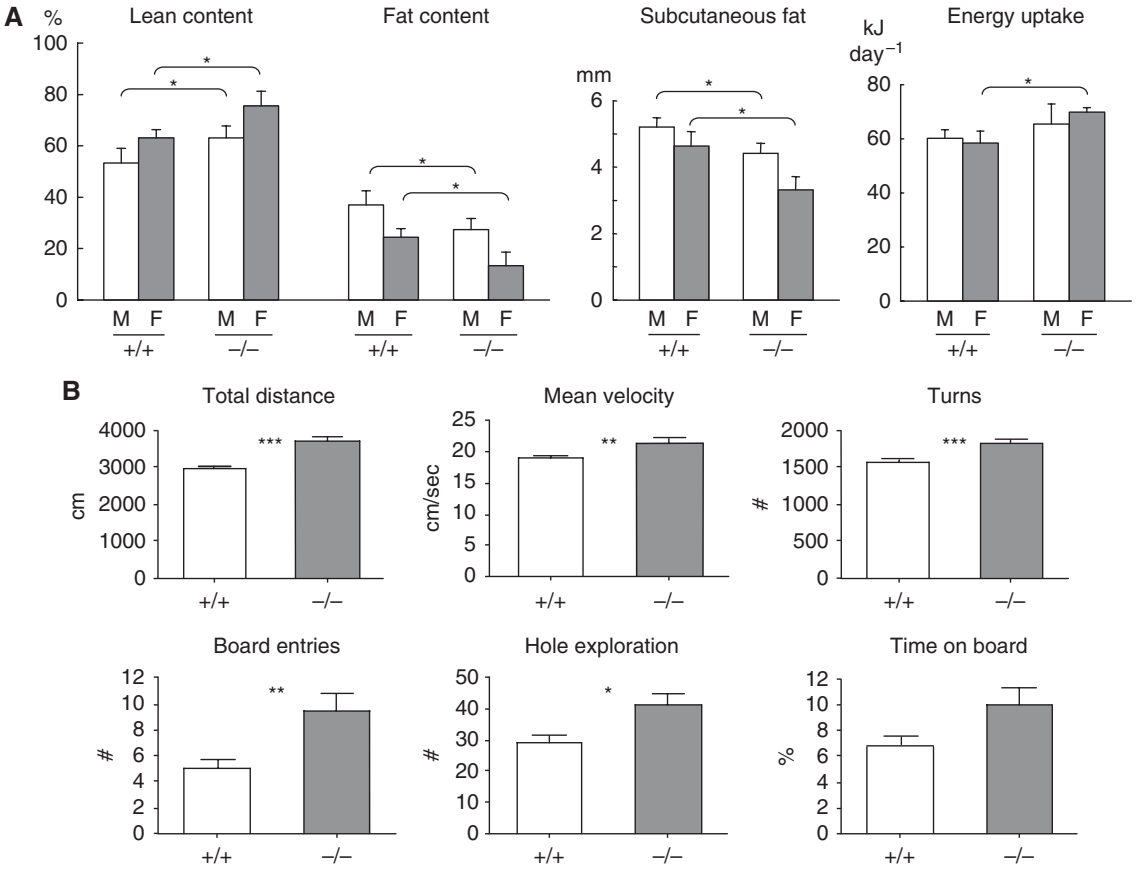


Figure 3 CIN85$^{\Delta ex2}$ knockout mice display metabolic and behavioural phenotypes. (**A**) CIN85$^{\Delta ex2}$ mutant mice display abnormalities in several metabolic parameters. Both male (M) and female (F) CIN85$^{\Delta ex2}$ knockout mice ($-/-$) showed a leaner body mass, as well as a decrease in total and subcutaneous fat content as compared with their wild-type littermates ($+/+$). Female CIN85$^{\Delta ex2}$ mice also showed a significantly increased energy uptake compared with their wild-type littermates ($+/+$). The graphs are based on the values in the table presented in Supplementary Figure S4B. $^{*}P<0.05$. (**B**) CIN85$^{\Delta ex2}$ knockout mice are hyperactive. Behavioural analysis of 8–10-week-old CIN85$^{\Delta ex2}$ ($-/-$) mice and wild-type ($+/+$) littermate controls ($n=22$–26 per genotype) by the modified hole-board test (described in detail in the Supplementary data). The CIN85$^{\Delta ex2}$ knockout mice showed significantly increased forward locomotor activity, speed, turning frequency, board entry frequency and hole exploration frequency, as compared with wild-type controls; $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$, $+/+$ versus $-/-$.

another member of the Cbl protein family, Cbl-b, which is involved in the regulation of long-term memory as well as short-term synaptic plasticity (Tan *et al*, 2006). This connection, together with the localization of CIN85 in dendritic spines of hippocampal neurons, led us to hypothesize that deficits in synaptic plasticity may be an underlying cause of the behavioural alterations observed in CIN85$^{\Delta ex2}$ mice. To address this question, we implemented established electrophysiological stimulation protocols (Freudenthal *et al*, 2004) to study synaptic plasticity in CIN85$^{\Delta ex2}$ mutants *in vivo*. Initially, we recorded field excitatory post-synaptic potentials (fEPSPs) in the dentate gyrus as a read-out of synaptic strength. In this assay, we found the synaptic response to be equally potentiated by tetanic stimulation in wild-type and CIN85$^{\Delta ex2}$ mice, thus indicating long-term potentiation (LTP) to be normal in the absence of CIN85 (Supplementary Figure S5A). Importantly, the stimulus–response curves of fEPSP slopes and population spikes, depicting synaptic strength and granule cell firing, respectively, were also unaltered in the mutants (Supplementary Figure S5B). Subsequently, we also analysed presynaptic short-term plasticity and GABAergic network inhibition by performing paired-pulse stimulation tests. However, in none of these assays did CIN85$^{\Delta ex2}$ knockout mice show any significant differences as compared with wild-type animals (Supplementary Figure S5C). We furthermore exposed the CIN85$^{\Delta ex2}$ mutants to learning and memory tests, and in line with the normal LTP and short-term plasticity, CIN85$^{\Delta ex2}$ knockout mice showed normal spatial learning in a water-maze place navigation task (Supplementary Figures S6A–C), as well as normal associative and contextual memory in a fear-conditioning task (Supplementary Figures S6A, D and E). Taken together, absence of CIN85 in the CNS does not cause any obvious abnormalities in learning and memory.

Dopamine receptor endocytosis is impaired in the absence of CIN85

The genetics behind behavioural traits such as locomotor activity and exploratory ambition is undoubtedly highly complex and involves a multitude of pathways, among which serotonergic, dopaminergic and noradrenergic signalling are frequently quoted (Pattij and Vanderschuren, 2008). On the basis of multiple studies reporting involvement of dopaminergic signalling in the regulation of movement, learning, reward-seeking behaviour and motivation (Yao *et al*, 2008), together with the rich clustering of dopamine receptors in dentritic spines (Missale *et al*, 1998; Gainetdinov *et al*, 2004; Viggiano *et al*, 2004; Zhu *et al*, 2004; Yao *et al*,

2008), we next investigated whether CIN85 could be involved in the regulation of dopamine signalling.

On the basis of the fact that increased levels of dopamine in the striatum have been correlated with locomotor hyperactivity phenotypes (Gainetdinov *et al*, 2004; Viggiano *et al*, 2004; Zhu *et al*, 2004), we first set out to analyse dopamine levels in the striatum of CIN85$^{\Delta ex2}$ knockout mice, using HPLC electro-chemical detection. Interestingly, we found that CIN85$^{\Delta ex2}$ knockout animals displayed significantly increased levels of dopamine in the striatum compared with wild-type animals (approximately 65% increase, Student's *t*-test, $P<0.05$) (Figure 4A). As elevation of dopamine levels should convey accumulation also of its metabolites, we additionally quantified the striatal concentrations of the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). In the process of dopamine degradation, dopamine is first converted into DOPAC and finally, after a series of subsequent events, into HVA, the end product in the dopamine metabolic pathway. The amounts of striatal DOPAC and HVA were higher in CIN85$^{\Delta ex2}$ knockout mice compared with wild-type littermates, consistent with the observed increase in dopamine levels (Figure 4A). Augmentation of dopamine levels may have multiple explanations, but in the light of earlier studies reporting important functions for CIN85 in the endocytic regulation of multiple cell surface receptors (see Introduction and Petrelli *et al*, 2002; Soubeyran *et al*, 2002; Kobayashi *et al*, 2004; Molfetta *et al*, 2005; Veiga and Cossart, 2005), we reasoned that the accumulation of dopamine in CIN85$^{\Delta ex2}$ mutant striata could be caused by defective internalization of dopamine receptors. Mammalian genomes have been reported to encode at least five different dopamine receptor sub-types, all belonging to the seven transmembrane G protein-coupled receptor (GPCR) superfamily (Yao *et al*, 2008). The receptors are entitled D1–D5 and fall into two distinct classes based on sequence and functional similarities,

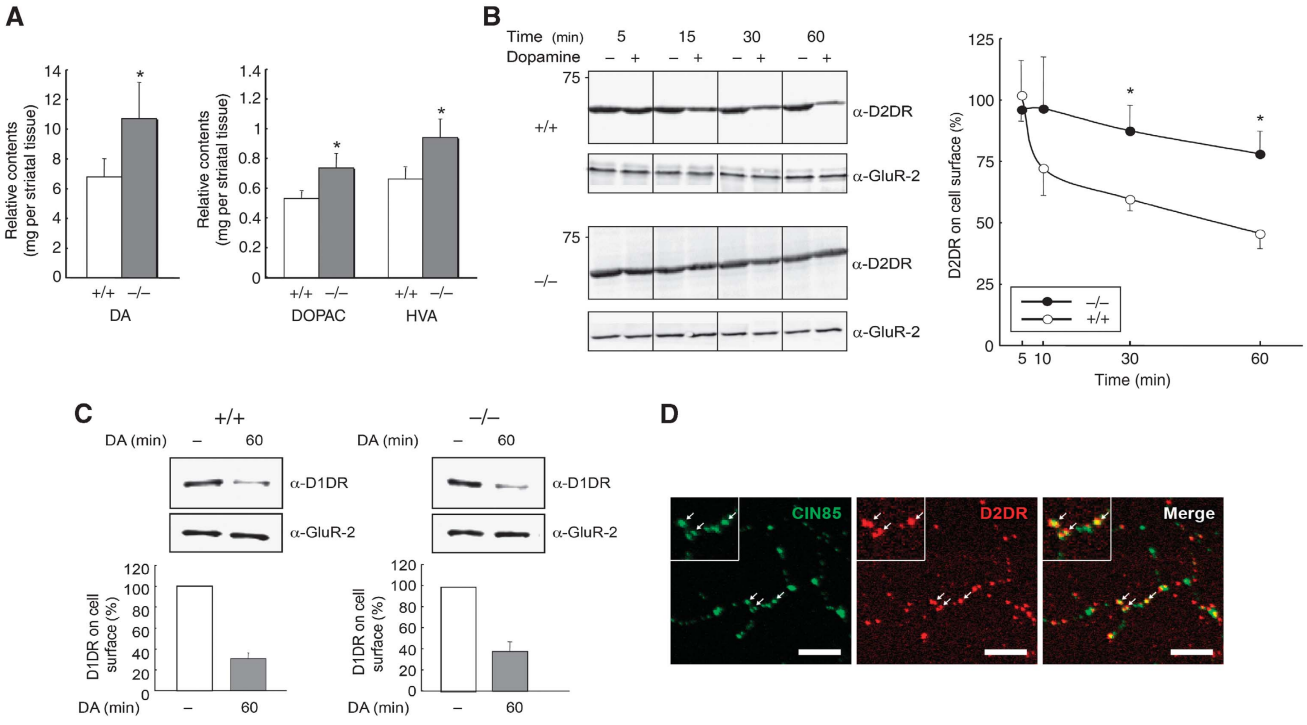


Figure 4 Impaired endocytic internalization of D2 dopamine receptors in mice lacking CIN85 expression in the CNS. (**A**) The levels of dopamine (DA) and its metabolites (3,4-dihydroxyphenylacetic acid, DOPAC and homovanillic acid, HVA) are increased in the striatum of CIN85$^{\Delta ex2}$ knockout mice. The contents of total striatal dopamine, DOPAC and HVA were determined by high performance liquid chromatography using an electro-chemical (HPLC-EC) detector as described in the Supplementary data. Differences between wild-type (+/+) and CIN85$^{\Delta ex2}$ knockout mice (−/−) were analysed using a Student's *t*-test, with the level of significance set at $^*P<0.05$. +/+: $n=4$; −/−: $n=4$. (**B**) D2 dopamine receptor endocytosis is impaired in striatal neurons deficient of CIN85. Representative immunoblots (left panel) and quantification (right panel) showing the amount of surface-associated D2 dopamine receptors (D2DR) in cultured striatal neurons after dopamine stimulation. Striatal neurons were treated with dopamine hydrochloride (3-hydroxytyramine, 20 μM) for the indicated times before biotinylation. The remaining cell surface proteins were subsequently isolated with avidin and analysed by western blotting. The quantification of surface-associated D2DRs after dopamine stimulation is normalized to and depicted as the reduction of band intensity as compared with non-stimulated cells at the indicated times. Differences between wild type (+/+) and CIN85$^{\Delta ex2}$ knockout (−/−) were analysed by ANOVA and Duncan multiple range test for *post hoc* between group comparisons, with the level of significance set at $^*P<0.05$. Glutamate receptor-2 (GluR-2) was used as an internal control. +/+: $n=3$; −/−: $n=3$. (**C**) Endocytosis of D1 dopamine receptors (D1DR) is not affected by loss of CIN85. Representative immunoblots and quantification showing the amount of surface D1DR in cultured striatal neurons before and after dopamine stimulation. Striatal neurons were treated with dopamine hydrochloride (20 μM) for 1 h before biotinylation. The remaining cell surface proteins were subsequently isolated with avidin and analysed by western blotting. D1DRs of cultured striatal neurons from wild-type (+/+) and CIN85$^{\Delta ex2}$ knockout mice (−/−) were internalized 69 ± 5.7% and 63 ± 8.8%, respectively. Glutamate receptor-2 (GluR-2) was used as an internal control. +/+: $n=3$; −/−: $n=3$. (**D**) CIN85 co-clusters with D2 dopamine receptors (D2DRs) at punctuate synapse-like structures in primary rat striatal neurons. Cells were fixed and stained with antibodies against CIN85 (SETA antibody, green) and D2DRs (red). Scale bars: 5 μm.

the D1-like (D1 and D5) and D2-like (D2, D3, D4) dopamine receptors. Whereas D1-like receptors are highly expressed in the prefrontal cortex, D2-like receptors, in particular the D2 dopamine receptor (D2DR) (Missale *et al*, 1998; Gainetdinov *et al*, 2004), show stronger expression in the striatum and nucleus accumbens (Arias-Carrion and Poppel, 2007). Even though D1 dopamine receptors (D1DRs) are also expressed in the striatum, D1DR and D2DR are in general segregated into distinct striatal neurons. Neurons expressing both receptor types are rare (Missale *et al*, 1998). Given the intriguing expression pattern of dopamine receptors, we were interested to analyse whether removal of CIN85-l and -xl isoforms in the striatum affects the endocytosis of D1DR and/or D2DR dopamine receptors. For this purpose, we used biotinylated D1 and D2 dopamine receptors to perform internalization assays in cultured primary striatal neurons from wild-type and CIN85$^{\Delta ex2}$ knockout mice, allowing us to follow the uptake of either D1DR or D2DR after dopamine stimulation. Interestingly, we detected diverging effects on the endocytosis of D1DR versus D2DR. When stimulating the neurons with dopamine for 5, 15, 30 and 60 min, neurons derived from wild-type animals rapidly decreased the levels of D2DRs dopamine receptors on the cell surface, whereas the levels of cell surface-associated D2DRs in neurons from CIN85$^{\Delta ex2}$ knockout mice remained high throughout the time course, depicting a function for CIN85 in D2DR internalization (Figure 4B). Specifically, after 15, 30 and 60 min of stimulation, striatal neurons from CIN85$^{\Delta ex2}$ knockout mice still maintained high levels of surface D2DRs (96.5, 87.5 and 78.1%, respectively), as compared with wild-type neurons, which displayed significantly reduced levels (72.3, 59.7 and 45.1%, respectively) ($P<0.05$) (Figure 4B). In contrast to D2DRs, internalization of D1DRs occurred at comparable levels in striatal neurons from wild-type and CIN85$^{\Delta ex2}$ knockout mice (Figure 4C). In both experiments, the level of an unrelated receptor (Glutamate receptor-2) was used as an internal control and did not change throughout the time course. To confirm the involvement of CIN85 in D2DR internalization, we additionally performed a receptor-ligand-binding assay, in which the binding of [^{3}H]spiperone, a tritium-labelled D2DR antagonist, to membrane fractions of striatal extracts from wild-type and CIN85$^{\Delta ex2}$ knockout mice was analysed. In agreement with the earlier experiment, striatal neurons from CIN85$^{\Delta ex2}$ knockout mice displayed increased levels of cell surface-associated D2DRs after dopamine stimulation (Supplementary Figure S7). Whereas wild-type neurons showed a 50% reduction of D2DR at the cell surface after 60 min of dopamine treatment, CIN85$^{\Delta ex2}$ knockout neurons retained 80% of cell surface-associated D2DR. Taken together, these results strongly indicate that lack of CIN85-xl and CIN85-l leads to impaired D2DR internalization, and in extension that CIN85 specifically regulates D2DR, but not D1DR, endocytosis in striatal neurons. Consistent with these findings, we found that CIN85 and D2DRs co-clustered in punctuate, synapse-like structures in dendrites of primary striatal neurons (Figure 4D).

CIN85$^{\Delta ex2}$ mice show altered behavioural responses to dopamine agonists and antagonists

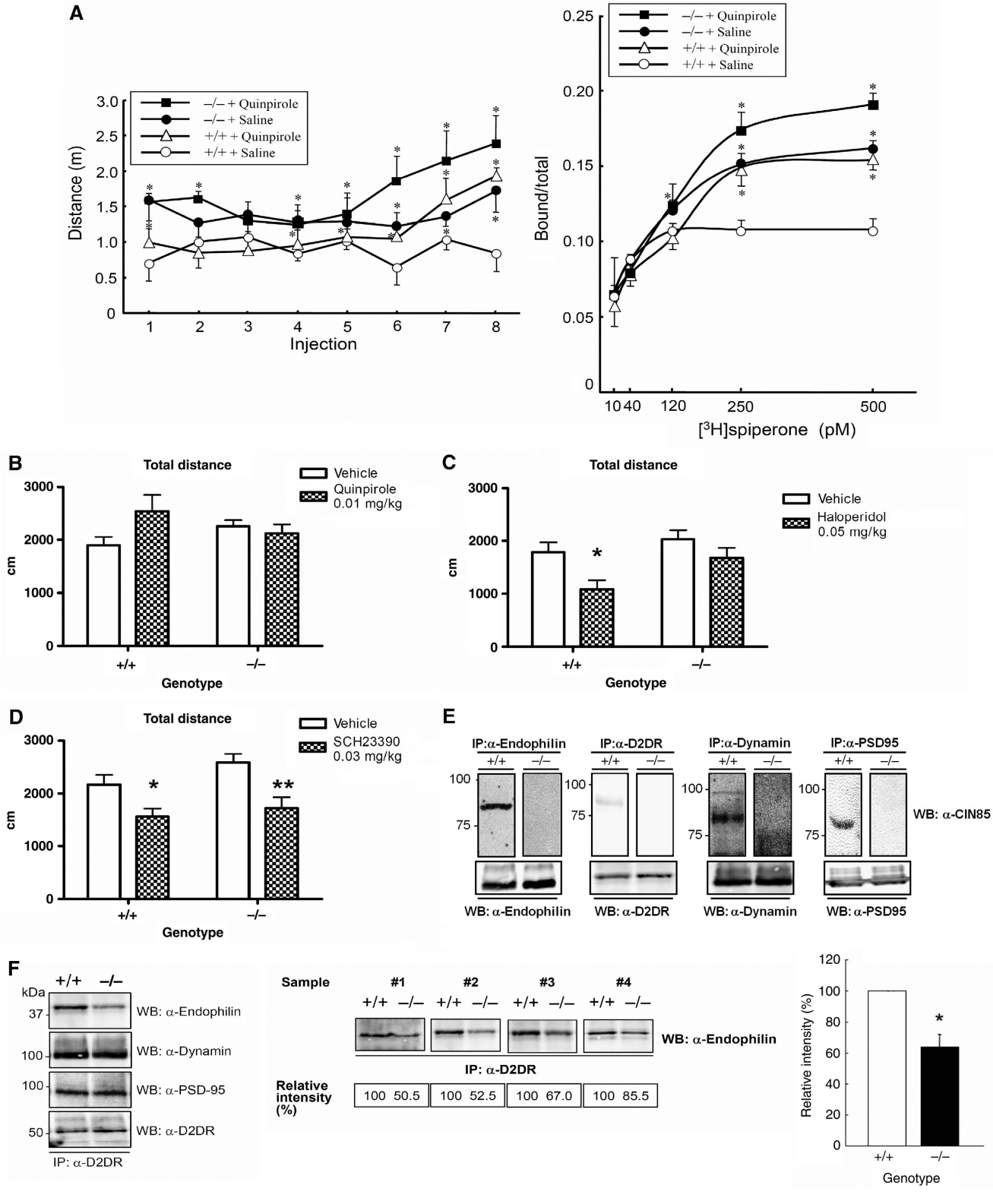
To investigate the connection between CIN85 and the D2DR under physiological conditions, we continued our studies by treating animals with the selective D2DR agonist quinpirole (or saline, as control). In both wild-type and CIN85$^{\Delta ex2}$ knockout mice repeated injections of quinpirole (0.5 mg/kg, s.c.) induced increased physical activity, manifested as an almost two-fold increase in distance travelled after eight injections of quinpirole compared with saline-injected control animals of both genotypes (Figure 5A, left panel). Interestingly, wild-type animals increased their locomotor activity after quinpirole treatment to similar levels as CIN85$^{\Delta ex2}$ knockout mice under saline-treated conditions (Figure 5A, left panel). In the light of earlier reports, this effect may be caused by a quinpirole-induced behavioural sensitization and increased dopamine receptor density in the striatum of treated mice (Culver *et al*, 2008). To test this hypothesis, we monitored the binding of [^{3}H]spiperone to D2DRs in striatal extracts prepared from mice after the final treatment and completion of the locomotor activity recordings. Indeed, the [^{3}H]spiperone binding was 36% higher in quinpirole-sensitized wild-type mice compared with saline-injected controls ($P<0.05$). In CIN85$^{\Delta ex2}$ knockout mice, the [^{3}H]spiperone binding was 45 and 73% higher in saline- and quinpirole-treated animals, respectively, when compared with wild-type mice treated with saline ($P<0.05$) (Figure 5A, right panel). These data show that the D2DR levels in the striatum are increased in CIN85$^{\Delta ex2}$ knockout mice *per se* and that sensitization to the D2DR agonist quinpirole increases the D2DR levels even further. In all cases, the level of striatal D2DR was thus strongly correlated with the intensity of the locomotor activity.

To further address the physiological relevance of CIN85 in dopaminergic neurotransmission, we investigated the effect of quinpirole and of haloperidol, a pharmacological inhibitor of dopamine receptors, on the behaviour of CIN85$^{\Delta ex2}$ knockout mice. At low doses, haloperidol is a selective antagonist for D2DR, whereas higher doses have been reported to act more promiscuously and target also D1DRs for inhibition (Leysen *et al*, 1988). Interestingly, we found that CIN85$^{\Delta ex2}$ animals were significantly less sensitive to low doses of quinpirole or haloperidol, compared with wild-type littermates (Figures 5B and C). Whereas wild-type mice exposed to a threshold dose of quinpirole (0.01 mg/kg) showed increased (approximately 34%) locomotor activity as indicated by total distance travelled (Figure 5B), CIN85$^{\Delta ex2}$ mutants seemed to be unaffected (genotype $\times$ treatment interaction: $F_{(1,7)}=4.17$, $P<0.05$). Correspondingly, wild-type control animals displayed significantly reduced locomotor activity (approximately 40%) when treated with a low dose of haloperidol (0.05 mg/kg) (treatment effect: $F_{(1,1)}=8.51$, $P<0.01$, Bonferroni's post-test vehicle versus haloperidol for wild type: $t=2.847$, $P<0.05$), whereas in contrast, CIN85$^{\Delta ex2}$ mutants did not (Bonferroni's post-test vehicle versus haloperidol for CIN85$^{\Delta ex2}$ mutants: $t=1.331$, NS) (Figure 5C). Higher doses of haloperidol (0.25 and 0.5 mg/kg) suppressed locomotor activity to a similar extent in both genotypes (data not shown). In contrast, treatment of wild-type and CIN85$^{\Delta ex2}$ mutants with a low dose of the D1-selective dopamine receptor antagonist SCH23390 (0.03 mg/kg; Figure 5D) yielded comparable suppression of locomotor activity in both genotypes (treatment effect: $F_{(1,1)}=16.89$, $P<0.001$, Bonferroni's post-tests vehicle versus SCH23390 for wild type: $t=2.365$, $P<0.05$, and for CIN85$^{\Delta ex2}$ mutants: $t=3.459$, $P<0.01$; genotype $\times$ treatment interaction: $F_{(1,43)}=0.47$, NS). Importantly, the effect produced

by the chosen dose of the D1-ligand SCH23390 was in the same range as that of the two D2-ligands, that is 0.03 mg/kg SCH23390 reduced locomotor activity in wild-type mice by 28% (Figure 5D). Taken together, these findings reinforce the notion that CIN85 has a specific function in D2-mediated, but not in D1-mediated processes *in vivo*.

Reduced complex formation between endophilins and D2DRs in CIN85$^{\Delta ex2}$ knockout mice

During receptor tyrosine kinase (RTK)-mediated endocytosis, CIN85 is targeted to activated receptors through the E3 ubiquitin ligase Cbl. Here, CIN85 functions to recruit components of the endocytic machinery that ultimately induces



receptor internalization (Petrelli *et al*, 2002; Soubeyran *et al*, 2002). To investigate whether CIN85 has a similar function during D2DR endocytosis, we analysed whether CIN85 is associated with endocytic protein complexes in synaptosome fractions prepared from mouse striata, focusing on endocytic proteins earlier implicated either to interact with CIN85 or to have a function in dopamine receptor endocytosis. Similar to other cell surface receptors, we found that endogenous CIN85 co-immunoprecipitates with the D2DR receptor itself, as well as with the endocytic proteins endophilin and dynamin in samples from wild-type animals (Figure 5E). Interestingly, we also found CIN85 to associate with PSD-95, a prototypical scaffolding protein localized at post-synaptic densities, which has been shown to facilitate constitutive endocytosis of dopamine receptors (Figure 5E) (Zhang *et al*, 2007). As expected, neither of these interactions was observed in CIN85$^{\Delta ex2}$ knockout striata (Figure 5E).

Knowing that CIN85 is associated with several endocytic regulators, we next examined how loss of CIN85 affects the complex formation between these components and D2DRs. Again, we performed co-immunoprecipitation experiments in synaptosomal fractions from mouse striata. When concurrently analysing wild-type and CIN85$^{\Delta ex2}$ knockout preparations, it was clear that in the absence of CIN85, the complex formation of endophilin with D2DRs was strongly reduced, whereas the association of D2DRs with dynamin and PSD-95 was unaltered (Figure 5F, left panel). Quantification of the binding of endophilin to D2DRs in wild-type and CIN85$^{\Delta ex2}$ striatal synaptosome fractions from four independent experiments showed that the interaction between D2DR and endophilin in CIN85$^{\Delta ex2}$ knockout striata is significantly decreased to about 65% of the interaction observed in wild-type samples (Student's *t*-test, $P<0.05$) (Figure 5F, middle and right panels). We thus conclude that CIN85 has a function in engaging endophilins in D2DR complexes, thereby promoting receptor endocytosis.

Finally, we attempted to uncover the mechanism by which CIN85 may be recruited to dopamine receptor complexes. We could not detect a direct binding between dopamine receptors and CIN85 in GST overlay assays (data not shown), suggesting that the interaction is likely to be mediated through an intermediate mechanism, possibly by recruitment through one or several of the many dopamine receptor-interacting proteins (DRIPs) (Kabbani and Levenson, 2007; Yao *et al*, 2008). Whereas we were unable to detect an interaction between D2DRs and c-Cbl/Cbl-b (data not shown), we have found CIN85 to interact with p62 in GST pull-down and co-immunoprecipitation assays (Supplementary Figure S8). This is highly interesting in the light of a recent report describing p62 as a novel DRIP, specifically involved in the trafficking of D2DRs (Kim *et al*, 2008).

Discussion

CIN85 regulates locomotor and exploratory behaviour

In this study, we lay down an important piece of the puzzle regarding the regulation of dopamine receptor signalling circuits in the CNS. By conventional methods, we have generated mice deficient of CIN85 expression in the brain and thus concluded that loss of CIN85 gives rise to hyperactive phenotypes. In wild-type neurons, CIN85 is enriched in dendritic spines and it clearly has a crucial function in stabilizing endophilin binding to D2DRs in the striatum. In consequence, absence of CIN85 gives rise to insufficient endocytic internalization of D2DRs after dopamine stimulation, causing increased striatal dopamine receptor levels, which can, at least in part, explain the enhanced locomotor and exploratory behaviour we observe in the CIN85$^{\Delta ex2}$ mice.

Figure 5 Defective complex formation between endophilins and D2 dopamine receptors in CIN85$^{\Delta ex2}$ knockouts. (**A**) Locomotor responses (left panel) and [^{3}H]spiperone binding (right panel) of quinpirole-treated mice. Mice of the indicated genotypes were repeatedly injected with quinpirole (0.5 mg/kg, s.c.) or saline in 48 h intervals until a total of eight injections were completed as described in the Supplementary data. Immediately after each injection, the locomotor activity was measured for 30 min using an actimeter. The left panel shows the distance travelled (m) during the last 5 min of the 30-min testing period. Data are presented as the mean ± s.e.m. ($n=3$). The difference between saline-injected wild types and the other genotypes/treatments were analysed by ANOVA and Student's *t*-test, with the level of significance set at $^*P<0.05$. Right panel: After the final measurement of locomotor activity, the membrane fraction of the striatum (12.5 μg/reaction) was prepared and incubated with [^{3}H]spiperone. Concentrations of [^{3}H]spiperone ranging from 10 to 500 pM were used, and non-specific binding was determined in the presence of D-butaclamol. Data are presented as the mean ± s.e.m. ($n=3$). The differences between saline-injected wild types and the other genotypes/treatments were analysed by ANOVA and Duncan multiple range test for *post hoc* between group comparisons, with the level of significance set at $^*P<0.05$. (**B**) Total distance travelled after a threshold dose of quinpirole; 19–26-week-old CIN85$^{\Delta ex2}$ (−/−) and wild-type (+/+) littermate control mice ($n=10$–15 per genotype) were subjected to the modified hole-board test 30 min after quinpirole injection (0 or 0.01 mg/kg i.p.) as described in the Supplementary data. (**C**) Total distance travelled after a low dose of haloperidol; 14–36-week-old CIN85$^{\Delta ex2}$ (−/−) and wild-type (+/+) littermate control mice ($n=10$–13 per genotype) were subjected to the modified hole-board test 30 min after haloperidol injection (0 or 0.05 mg/kg i.p.) as described in the Supplementary data. $^*P<0.05$, vehicle versus haloperidol, Bonferroni's post-tests. (**D**) Total distance travelled after a low dose SCH23390; 10–11-week-old CIN85$^{\Delta ex2}$ (−/−) and wild-type (+/+) littermate control mice ($n=10$–12 per genotype) were subjected to the modified hole-board test 30 min after SCH23390 injection (0 or 0.03 mg/kg i.p.) as described in the Supplementary data. $^*P<0.05$, $^{**}P<0.01$, vehicle versus SCH23390, Bonferroni's post-tests. (**E**) Synaptosome fractions were prepared from wild-type and CIN85$^{\Delta ex2}$ knockout mouse striata by sucrose density-gradient centrifugation. In total, 25 μg of fractionated protein was analysed by immunoprecipitation (IP) followed by western blotting (WB) using the indicated antibodies. CIN85 co-immunoprecipitates with D2DRs, the endocytic proteins endophilin and dynamin, as well as the post-synaptic density protein PSD-95, in wild type, but not in CIN85$^{\Delta ex2}$ knockout samples. The upper band evident in the dynamin IP may be CIN85-xl. (**F**) The level of endophilin in complex with the D2DR is decreased in the striatal synaptosome fractions in CIN85$^{\Delta ex2}$ knockout mice. Synaptosomes of wild-type (+/+) and CIN85$^{\Delta ex2}$ knockout (−/−) mouse striata were prepared as in (**E**) and subjected to IP with anti-D2DR antibodies and subsequent immunoblot analysis with antibodies against endophilin, dynamin, PSD-95 and the D2DR (left and middle panels). Western blots from four independent experiments are shown in the middle panel. Relative intensities of the endophilin levels in CIN85$^{\Delta ex2}$ knock outs compared with wild type for each of the four experiments are indicated. A graph depicting the mean value of the relative endophilin intensities in CIN85$^{\Delta ex2}$ knockout (−/−) compared with wild-type (+/+) mice from the four experiments is shown in the right panel. The value is presented as mean ± s.e. Differences between wild-type (+/+) and CIN85$^{\Delta ex2}$ knockout mice (−/−) were analysed using a Student's *t*-test, with the level of significance set at $^*P<0.05$. +/+: $n=4$; −/−: $n=4$.

The involvement of dopaminergic signalling in the regulation of movement, emotion and reward feelings is well established (Arias-Carrion and Poppel, 2007). In agreement, aberrations in these pathways have been strongly linked to various neurological disorders, including Parkinson's disease, schizophrenia, ADHD and Huntington's disease (Gainetdinov *et al*, 2004; Arias-Carrion and Poppel, 2007). The molecular defects underlying these pathologies have not been fully characterized, but may include alterations in the expression levels of dopamine ligands and/or receptors, as well as defects in downstream signalling events (Bellgrove and Mattingley, 2008; O'Connell *et al*, 2008). Interestingly, the hyperactive behaviour evoked by alterations in other pathways (such as glutamatergic and serotonergic cascades) or by the action of chemical substances (such as haloperidol and clozapine), in many cases, also indirectly affects dopaminergic signalling. Specifically, in several such cases, the described defect or compound has been reported to stimulate increased levels of high-affinity state D2DRs ($D2^{High}$) in the striatum and nucleus accumbens (Seeman *et al*, 2006).

CIN85 regulates dopamine receptor endocytosis in the striatum

This report provides the first study of a mammal in which loss of CIN85 is analysed *in vivo*. Specifically, we have generated mice in which the two CIN85 isoforms expressed in the CNS, CIN85-xl and -l, have been removed. Most probably because of the presence of CD2AP, together with multiple alternative CIN85 isoforms expressed in other tissues, $CIN85^{\Delta ex2}$ mice are viable and fertile. This feature has thus enabled us to identify tissue-specific functions of CIN85 in the brain, in the regulation of proper neurotransmission.

Under physiological conditions, endocytic internalization of dopamine receptors is a crucial means by which neurons, in response to dopamine stimulation, can adjust their excitability potential, by either degrading or recycling receptors back to the plasma membrane (von Zastrow, 2003). A stringent control of this dynamic trafficking of dopamine receptors, as well as receptors for other neurotransmitter substances, is essential to ensure accurate transmission of neuronal information and proper regulation of synaptic plasticity (Gainetdinov *et al*, 2004). We have discovered that CIN85 affects the endocytosis of D2DR, but not D1DR, in primary striatal neurons. The resulting increase of surface-associated D2DRs in $CIN85^{\Delta ex2}$ knockout mouse striatal neurons and ensuing hyperactivity phenotype are in line with earlier findings, showing that activation of post-synaptic D2DRs results in increased locomotor activity and that D2DR knockout mice display reduced spontaneous movement (Jackson and Westlind-Danielsson, 1994; Baik *et al*, 1995). Importantly, activation of D1DRs, on the other hand, has little or no effect on locomotor activity (Jackson and Westlind-Danielsson, 1994). The specificity of CIN85 involvement in D2DR endocytosis could have multiple explanations. Either it may be a result of the restricted expression of D1DR and D2DR in distinct striatal neuron populations (Missale *et al*, 1998) causing a cell-specific positioning of CIN85 preferentially in D2DR-expressing neurons, or simply that CIN85 performs diverse functions during the internalization of different receptor types. Indeed, separate endocytic machineries and routes have been reported for D1DR versus D2DR (Vickery and von Zastrow, 1999; Iizuka *et al*, 2007; Kabbani and Levenson, 2007). Interestingly, we find CIN85 co-clustered with D2DRs in synapse-like structures in primary striatal neurons. Taken together, our data imply a specific function for CIN85 in the regulation of D2DR endocytosis in striatal neurons.

CIN85 facilitates complex formation between endophilins and D2DRs

Molecularly, we have observed that CIN85 displays important scaffolding functions in the complex formation between endophilins and D2DRs in striatal neurons, a finding that is in agreement with earlier discoveries showing a direct interaction between endophilins and CIN85 during the initiation of receptor internalization (Petrelli *et al*, 2002; Soubeyran *et al*, 2002). The endophilins constitute a family of proteins with well-established functions in the process of endocytosis, during which they are important both for the recruitment of endocytic machinery components to sites of activated receptors, as well as for the induction of membrane curvature, a function mediated through their N-BAR domains (Gallop *et al*, 2006; Masuda *et al*, 2006). Insufficient targeting of endophilins to dopamine receptor complexes could, therefore, result in accumulation of activated receptors at the plasma membrane of neurons, thereby enhancing dopaminergic signalling activities, and in extension cause aberrations in behaviour.

Importantly, this study provides the first evidence that CIN85/endophilin complexes are involved in the endocytosis of a member of the GPCR family. In the light of this finding, it is interesting to remark that the arrestins, another family of adaptor proteins with well-established functions in GPCR endocytosis and trafficking, also have a function in internalization of RTKs (Lefkowitz *et al*, 2006), thus exposing both CIN85 and arrestins as adaptors with the capacity to regulate the endocytosis and trafficking of a broad range of receptor types, including both RTKs and GPCRs. Despite extensive efforts, we have not been successful in clearly establishing the mechanism of CIN85 recruitment to dopamine receptor complexes. The interaction is likely to be mediated through an intermediate mechanism, possibly by recruitment through one or several of the many DRIPs, such as p62, which we indeed have found in complex with CIN85 (Kabbani and Levenson, 2007; Yao *et al*, 2008). The multiplicity of possible interacting partners and the redundancy between distinct important pathways could explain why the loss of CIN85 function can have deleterious effects on a selection of receptor types in specific tissues, whereas other classes of receptors in contrast seem to be unaffected (Petrelli *et al*, 2002; Soubeyran *et al*, 2002; Kobayashi *et al*, 2004). Indeed, a function of CIN85 specifically in the regulation of D2DR activity would be in agreement with the reported hypoactivity (i.e. reduced locomotor activity) displayed by D2DR knockout mice, which is in contrast to the hyperactivity, which has been observed in mice deficient of other D2-like dopamine receptor sub-types (Holmes *et al*, 2004).

Increased D2DR levels in the absence of CIN85 correlate with hyperactive behaviour and altered metabolism

During our extensive physiological examination of the $CIN85^{\Delta ex2}$ mice, we were interested to find that mice lacking striatal expression of CIN85 display a lean body composition,

even though consuming more food than wild-type animals. However, in contrast to c-Cbl knockouts in which the anomalous lean body composition is accompanied by alterations in insulin metabolism, CIN85$^{\Delta ex2}$ mice did not show any significant improvements in conventional glucose tolerance tests (data not shown). The explanation for this phenotypical trait may instead be directly linked to the observed alterations in dopaminergic activity, given the earlier reported involvement of D2DR-mediated signalling in the regulation of appetite, energy intake and obesity. Multiple studies have shown a correlation between striatal D2DR expression levels and body composition and have associated low D2DR with obesity (Epstein *et al*, 2007). A recent report has furthermore showed a strong link between certain D2DR allelic variations and obesity, suggesting that individuals with certain genotypes resulting in dopaminergic hypofunction are prone to obesity (Stice *et al*, 2008). Increased dopaminergic signalling is, therefore, in agreement with the slim appearance of CIN85$^{\Delta ex2}$ knockouts.

Concluding remarks

Notably, a function of CIN85 at neuronal synapses is well in line with the reported importance of CD2AP/CIN85 at immunological synapses in T cells, as well as in the slit diaphragm in kidney glomeruli, given the structural as well as functional similarities between neuronal synapses and these specialized cell surface contacts (Shih *et al*, 1999; Dustin and Colman, 2002). In conclusion, CIN85 is a novel regulator of D2DR endocytosis in striatal neurons, involved in controlling locomotor and exploratory behaviour in mice.

Materials and methods

Generation of the CIN85$^{\Delta ex2}$ knockout mice

The removal of exon 2 from the mouse CIN85 locus by homologous recombination and the generation of the CIN85$^{\Delta ex2}$ knockout mice are described in the Supplementary data.

Phenotypic analyses of behaviour, metabolism and other parameters

See the Supplementary data.

Immunofluorescence of primary neurons

See the Supplementary data.

Isolation of synaptosomes and post-synaptic density fractions

See the Supplementary data.

Dopamine receptor biotinylation and endocytosis assay

See the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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