Cell adhesion molecule close homolog of L1 binds to the dopamine receptor D2 and inhibits the internalization of its short isoform

Agnieszka Kotarska1 | Luciana Fernandes1 | Ralf Kleene1 | Melitta Schachner2,3

1Zentrum für Molekulare Neurobiologie, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany
2Center for Neuroscience, Shantou University Medical College, Shantou, China
3Keck Center for Collaborative Neuroscience and Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ, USA

Correspondence
Melitta Schachner, Center for Neuroscience, Shantou University Medical College, 22 Xin Ling Road, Shantou, Guangdong 515041, China.
Email: schachner@stu.edu.cn

Funding information
Li-Kashing Foundation

Abstract
Cell adhesion molecule close homolog of L1 (CHL1) and the dopamine receptor D2 (DRD2) are associated with psychiatric and mental disorders. We here show that DRD2 interacts with CHL1 in mouse brain, as evidenced by co-immunostaining, proximity ligation assay, co-immunoprecipitation, and pull-down assay with recombinant extracellular CHL1 domain fused to Fc (CHL1-Fc). Direct binding of CHL1-Fc to the first extracellular loop of DRD2 was shown by ELISA. Using HEK cells transfected to co-express CHL1 and the short (DRD2-S) or long (DRD2-L) DRD2 isoforms, co-localization of CHL1 and both isoforms was observed by immunostaining and proximity ligation assay. Moreover, CHL1 inhibited agonist-triggered internalization of DRD2-S. Proximity ligation assay showed close interaction between CHL1 and DRD2 in neurons expressing dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP32) or tyrosine hydroxylase (TH) in tissue sections of adult mouse striatum. In cultures of striatum or ventral midbrain, CHL1 was also closely associated with DRD2 in DARPP32- or TH-immunopositive cells, respectively. In the dorsal striatum of CHL1-deficient mice, lower levels of DRD2 and phosphorylated TH were observed, when compared to wild-type littermates. In the ventral striatum of CHL1-deficient mice, levels of phosphorylated DARPP32 were reduced. We propose that CHL1 regulates DRD2-dependent presynaptic and postsynaptic functions.

KEYWORDS
monoaminergic receptors, neurotransmission, postsynaptic compartment, presynaptic compartment, striatum

Abbreviations: CHL1, cell adhesion molecule close homolog of L1; CHL1−/−, CHL1-deficient mice; CHL1+/+, wild-type littermates of CHL1-deficient mice; CHL1-Fc, fusion protein of the extracellular CHL1 domain and Fc; DARPP32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; DRD2, dopamine receptor D2; DRD2-L, long isoform of DRD2; DRD2-S, short isoform of DRD2; NCAM, neural cell adhesion molecule; NCAM-Fc, fusion protein of the extracellular NCAM domain and Fc; pSer40-TH, tyrosine hydroxylase phosphorylated at Ser40; pThr34-DARPP32, DARPP32 phosphorylated at Thr34; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.
INTRODUCTION

The dopaminergic system plays important roles in many nervous system functions regulating locomotion, cognition, and reward as well as social and emotional behaviors. Impaired dopaminergic signaling is implicated in several neurological and psychiatric disorders, such as Parkinson's disease, depression, schizophrenia, drug abuse, and neuroinflammation. 

Dopamine exerts its effect through dopamine receptors that can be divided into two subclasses: D1-like (D1 and D5) and D2-like (D2, D3, and D4). In particular, DRD2 plays a key role in dopaminergic transmission and is an important player in the pathogenesis of schizophrenia. As a result of alternative splicing, DRD2 exists in two isoforms: DRD2-S and DRD2-L which differ by 29 amino acids in the third cytoplasmic loop. DRD2 can function as a presynaptic autoreceptor to inhibit dopamine release upon activation. Both DRD2-L and DRD2-S function as autoreceptors, but display differences in trafficking and binding properties and control different signal transduction pathways.

Increasing evidence indicates that an adhesion molecule of the immunoglobulin superfamily and L1 subfamily, namely CHL1, is implicated in mental retardation and psychiatric disorders, such as schizophrenia and autism spectrum disorders. CHL1 deficiency in mice leads to impairments in synaptic transmission, long-term potentiation, working memory, and gating of sensorimotor information or prepulse inhibition of startle, which is one characteristic feature of schizophrenic patients. Constitutive and neurona1onal conditional ablation of CHL1 in mice leads to alterations in social and exploratory behaviors.

Since DRD2, on the one hand, and CHL1, on the other, are associated with psychiatric disorders, it seemed likely that a functional relationship exists between DRD2 and CHL1. Furthermore, based on evidence that neurotransmitter, cytoadhesion molecules and thus impinge on synaptic modifications not only during ontogenetic development but also in the adult, we set out to investigate CHL1 in its relation to neurotransmitter receptors. Here, we show that CHL1 and DRD2 form a functional complex and that CHL1 regulates internalization and thereby desensitization of this receptor.

MATERIALS AND METHODS

Animals

CHL1-deficient (CHL1−/−) mice had been back-crossed onto the C57BL/6J background for more than eight generations. CHL1−/− mice and their age-matched wild-type littermates (CHL1+/+) as well as C57BL/6J mice were bred and maintained at the Universitätsklinikum Hamburg-Eppendorf. Animals were housed at 25°C on a 12-h light/12-h dark cycle with ad libitum access to food and water. All animal experiments were approved by the local authorities of the State of Hamburg and conform to the guidelines set by the European Union.

Antibodies and reagents

The polyclonal rabbit antibodies against the extracellular domain of CHL1 and NCAM have been described. The polyclonal goat antibody against extracellular CHL1 epitopes (R and D Systems Cat# AF2147, RRID:AB_2079332) was from R&D Systems (Minneapolis, MN, USA). The mouse monoclonal antibody against DRD2 (Santa Cruz Biotechnology Cat# sc-5303, RRID:AB_668816), the rabbit polyclonal antibody against GAPDH (Santa Cruz Biotechnology Cat# sc-25778, RRID:AB_10167668), and Protein A/G agarose beads were from Santa Cruz Biotechnology (Heidelberg, Germany). The rabbit monoclonal antibody against DARPP32 (Cell Signaling Technology Cat# 2306, RRID:AB_823479) was from Cell Signaling Technology Europe (Frankfurt am Main, Germany) and the polyclonal rabbit antibody against tyrosine hydroxylase (TH) (Millipore Cat# AB152, RRID:AB_390204) was from Merck Chemicals (Darmstadt, Germany). The polyclonal rabbit antibodies against TH phosphorylated at Ser40 (pSer-TH) (Bio-Rad/AbD Serotec Cat# AHP912, RRID:AB_567401) and against DARPP32 phosphorylated at Thr34 (pThr34-DARPP32) (Bio-Rad/AbD Serotec Cat# AHP897, RRID:AB_566944) were from Bio-Rad (Puchheim, Germany). Horseradish peroxidase-conjugated and fluorescent dye-coupled secondary antibodies were from Dianova (Hamburg, Germany). Synthetic peptides comprising the extracellular DRD2 N-terminus (biotin-MDPN-LSWYDDDLERQNSRPFNGSEGKPRPHNYY-OH) or the first (biotin-LEVVGWKSRIHCD-OH), second (biotin-GLNNTDQNECIIANPA-OH), or third (biotin-NI-HCDNIPPVLYS-OH) extracellular DRD2 loop were from Schäfer-N (Copenhagen, Denmark). Fusion proteins comprising the extracellular domains of CHL1 or NCAM and the Fc fragment of human IgG (CHL1-Fc, NCAM-Fc) were produced by InVivo BioTech Services (Hennigsdorf, Germany) and have been described. The plasmid pCAG-EGFP (RRID:Addgene_89684) was from Addgene (Watertown, MA, USA) and the plasmids pcDNA3.1 with DRD2-L-coding cDNA (OHu23461) and pcDNA3.1 with DRD2-S-coding cDNA (OHu23557) were from GenScript, Piscataway, NJ, USA). Primers were from Metabion International (Planegg, Germany) and the In-Fusion kit was from Takara Bio Europe (Saint-Germain-en-Laye, France). Phosphatase and protease inhibitors, quinpirole, carrageenan, bovine serum albumin, fatty acids-free bovine serum albumin, and Duolink PLA products were from Sigma-Aldrich (Taufkirchen, Germany).
Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum, penicillin/streptomycin, and phosphate-buffered saline (PBS) were from PAN Biotech (Aidenbach, Germany). Lipofectamine LTX with Plus Reagent, the BCA Protein Assay Kit, Protein A magnetic beads, Sulfo-NHS-LC-Biotin, streptavidin-conjugated magnetic beads, o-phenylenediamine, and stable peroxidase buffer were from ThermoFisher Scientific (Darmstadt, Germany).

HEK293 cells (CLS Cat# 300192/p777_HEK293, RRID:CVCL_0045) were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Nonfat dry milk, cacodylate buffer, and mounting medium with DAPI were from Carl Roth (Karlsruhe, Germany) and enhanced chemiluminescence solutions from GE Healthcare Lifesciences (Marlborough, MA, USA). Tissue-Tek OCT Compound was from Sakura Finetek Europe (Alphen aan den Rijn, The Netherlands).

2.3 Cloning of plasmid constructs and transfection of HEK293 cells

The In-Fusion kit was used according to the manufacturer’s protocol for cloning of the expression vectors pCAG-DRD2-S, pCAG-DRD2-L, pCAG-DRD2-S/CHL1, and pCAG-DRD2-L/CHL1 that allow the expression of DRD2-S or DRD2-L alone or the simultaneous expression of CHL1 and DRD2-S or DRD2-L. Using the IRES-containing bicistronic vector pCAG-EGFP as template and the primers pCAG fw (5′-AGCGGCCGGCCGCCAGCACAGTGG-3′) and pCAG rev (5′-CGGGGCGAAGGCAACGCAGCGACT-3′) for PCR, part of the pCAG was amplified and used as a backbone for cloning of the expression vectors. pCAG-EGFP and the primers IRES fw (5′-ATGGTAATCGTGCGAGAGG-3′) and IRES-pCAG rev (5′-TGGCAATTCCATCATGGTTGTGGC CATATTATCAT-3′) or IRES-CHL1 rev (5′-TGGCGGCCGGCCGTGGTTGTGGCCATATTATCAT-3′) were exploited to amplify the IRES-coding cDNAs used for cloning of pCAG-DRD2-S and pCAG-DRD2-L or of pCAG-DRD2-S/CHL1 and pCAG-DRD2-L/CHL1, respectively. Using pcDNA3.1 carrying either DRD2-L-coding cDNA or DRD2-S-coding cDNA as template and the primers DRD2 fw (5′-GTTGCCTTCGCCCCGATGGATCCACTGAATCTGGTC-3′) and DRD2 rev (5′-TCGCACGATTACCATTCAGCAGTGGAGGATCTTCAG-3′), cDNAs coding for DRD2-L and DRD2-S were amplified. CHL1-coding pcDNA3 vector34 and the primers CHL1 fw (5′-ATGATGGAATTGCCATTATATGT-3′) and CHL1 rev (5′-TGGCGGCCGGCCCTTCATGCGAGTGGGAA-3′) were used for PCR amplification of CHL1-coding cDNA. The PCR products were combined accordingly and processed using the In-Fusion kit. The obtained plasmid constructs were amplified in Stellar competent *Escherichia coli* from the In-Fusion kit and used to transfect HEK293 cells (CRL-1573, ATCC) grown in DMEM containing 10% fetal bovine serum and 2% penicillin/streptomycin. Transfections were performed at a confluency of ~70% using Lipofectamine LTX with Plus Reagent according to the manufacturer’s protocol. Two days after transfection, cells were used for experiments.

2.4 Preparation of tissue homogenates and of HEK293 cell lysates

Brains or ventral and dorsal striata were homogenized on ice in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) containing phosphatase and protease inhibitors. Homogenates of dorsal and ventral striata were additionally subjected to 8 pulses for 30 seconds at >20 kHz on ice using a Kontes Micro Ultrasonic Cell Disrupter KT 50 (Vineland, NJ, USA).

HEK293 cells were incubated with lysis buffer containing protease and phosphatase inhibitors for 15 minutes on ice and then collected. Tissue homogenates and cell lysates were centrifuged for 10 minutes at 1000 g and 4°C followed by determination of proteins concentration using the BCA Protein Assay Kit.

2.5 Co-immunoprecipitation, pull-down assay, and Western blot analysis

For immunoprecipitation, whole-brain homogenates (~500 μg protein) were pre-cleared for 30 minutes at 4°C with Protein A/G agarose beads. Rabbit polyclonal anti-CHL1 or anti-NCAM antibodies (1:100) or mouse monoclonal anti-DRD2 antibody (5 μg/mL) was added to the pre-cleared homogenates and incubated for 1 hour at 4°C followed by overnight incubation at 4°C with Protein A/G agarose beads. Beads were washed twice at 4°C with the lysis buffer and once with PBS and boiled in sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, and 0.0625 M Tris-HCl, pH 6.8).

For pull-down, whole-brain homogenates (~500 μg protein) were first pre-cleared for 1 hour at 4°C with Protein A magnetic beads and then incubated with ~100 μg CHL1-Fc or NCAM-Fc and Protein A magnetic beads for 30 minutes at room temperature. Beads were washed three times at 4°C with lysis buffer and once with PBS. Bound proteins were eluted by boiling in sample buffer.

For Western blot analysis, proteins were transferred to nitrocellulose membrane (VWR, Darmstadt, Germany) after sodium dodecyl sulfate polyacrylamide gel electrophoresis. Membranes were blocked in 5% bovine serum albumin or
% nonfat dry milk in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris-Cl, pH 7.5) containing 0.05% Tween 20 and incubated overnight at 4°C with mouse monoclonal anti-DRD2 antibody (1:200), rabbit polyclonal anti-CHL1 antibody (1:1000), rabbit polyclonal anti-NCAM antibody (1:1000), goat polyclonal anti-CHL1 antibody (1:1000), rabbit polyclonal anti-GAPDH antibody (1:1000), rabbit polyclonal anti-TH antibody (1:1000), rabbit polyclonal anti-pSer40-TH (1:500), rabbit monoclonal anti-DARPP32 antibody (1:1000), or rabbit polyclonal anti-pThr34-DARPP32 antibody (1:200). After washing with TBS containing 0.05% Tween 20, the membranes were incubated at room temperature for 1 hour with horseradish peroxidase-conjugated secondary antibodies (1:10 000) and washed with TBS containing 0.05% Tween 20. Immunopositive bands were visualized by chemiluminescence detection at the ImageQuant LAS 4000 Mini biomolecular imager (GE Healthcare) after application of enhanced chemiluminescence solution to the membrane. Protein levels were determined using ImageJ.

2.6 | ELISA

High-binding 384-well plates (Corning, Amsterdam, The Netherlands) were coated overnight at 4°C with 25 µL solution of synthetic peptides comprising the extracellular DRD2 N-terminus (12 µg/mL) or the first, second, or third extracellular DRD2 loop (4.73 µg/mL). After washing with PBS and blocking at room temperature for 1 hour with 2% bovine serum albumin in PBS, the wells were incubated with different concentrations of CHL1-Fc and NCAM-Fc for 1 hour at room temperature followed by three washing steps with PBS. For detection of bound Fc proteins, horseradish peroxidase-conjugated anti-human antibody (1:2500) was applied to the wells for 1 hour at room temperature followed by washing and incubation with 0.5 mg/mL o-phenylenediamine in stable peroxidase buffer. The reaction was monitored and then stopped using 2.4 M H2SO4. Absorbance at 492 nm was determined on the µQuant microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA).

2.7 | Cell surface biotinylation

Transfected HEK293 cells were either incubated for 30 minutes with PBS or with 10 µM quinpirole in PBS prior to cell surface biotinylation or were directly subjected to cell surface biotinylation. The cells were rinsed twice with PBS and incubated on ice for 15 minutes with 0.5 mg/mL Sulfo-NHS-LC-Biotin in PBS. The cells were washed with PBS, incubated for 5 minutes on ice with 20 mM glycine in PBS to quench unreacted reagent, washed with PBS and treated with lysis buffer. Aliquots of the lysates were collected and served as input control. The rests of the lysates were incubated overnight with streptavidin-conjugated magnetic beads. The beads were washed once with lysis buffer and three times with PBS. Bound proteins were eluted by boiling in sample buffer.

2.8 | Immunofluorescent detection of cell surface and internalized DRD2

Transfected HEK293 cells on glass coverslips coated with poly-L-lysine were incubated with serum-free DMEM containing 0.5% fatty acids-free bovine serum albumin and mouse monoclonal anti-DRD2 antibody (1:500) for 1 hour at 4°C. The cells were rinsed twice with serum-free DMEM and stimulated with 10 µM quinpirole in serum-free DMEM for 30 minutes at 37°C. The cells were fixed with 4% formaldehyde in PBS for 5 minutes at room temperature, rinsed with PBS, and blocked in 5% bovine serum albumin in PBS for 1 hour at room temperature. The cells were then incubated with Cy3-conjugated anti-mouse antibody (1:500) for 5 hour at room temperature to label DRD2 at the cell surface. After washing with PBS and a second fixation with 4% formaldehyde in PBS for 5 minutes at room temperature, cells were subjected to blocking and permeabilization by applying 5% bovine serum albumin in PBS containing 0.1% Triton X-100 for 30 minutes at room temperature. The permeabilized cells were incubated overnight with the Cy2-conjugated anti-mouse antibody (1:500) at 4°C to label internalized DRD2. After washing the cells with PBS, the coverslips were mounted on Superfrost Plus glass slides (ThermoFisher Scientific) using mounting medium with DAPI.

2.9 | Fixation of tissue and cultured cells, cryosectioning, and immunofluorescent staining

Mice were perfused with 4% formaldehyde in 0.1 M sodium cacodylate buffer or PBS. The brains were removed and post-fixed in the same fixation solution for 1 day or 2 days at 4°C, cryoprotected at 4°C for 3 days in 15% or 30% sucrose diluted in 0.1 M sodium cacodylate buffer or PBS, shock-frozen in 2-methyl-butane at −80°C and stored at −80°C until use. Before cryosectioning (Leica CM3050 cryostat, Leica Instruments, Wetzlar, Germany or CryoStar NX70 Kryoostat, Thermo Scientific), the brains were immersed in Tissue-Tek OCT Compound, 20 µm-thick sections were collected on Superfrost Plus glass slides (ThermoFisher Scientific), dried at room temperature, and stored at −20°C. Alternatively, 40 µm-thick serial sections were collected and stored free-floating in PBS with 0.02% sodium azide at 4°C until use. Before immunostaining, the mounted or free-floating sections
were subjected to antigen retrieval for 30 minutes at 80°C in 2.94 mg/mL tri-sodium citrate dihydrate, pH 9.0, cooled down to room temperature, and then transferred to blocking buffer (5% donkey serum, 0.2% Triton X-100, 0.02% sodium azide in PBS).

Transfected HEK293 cells on poly-L-lysine-coated glass slides (Millipore EZ Slides, Merck Chemicals) were washed with PBS and either directly fixed with 4% formaldehyde in PBS or fixed after preincubation at 37°C for 15 minutes with PBS or 50 µg/mL CHL1-Fc in PBS. Cells were washed with PBS and incubated for 1 hour at room temperature with blocking serum. Cultured cells from embryonic midbrain and striatum were fixed with 4% formaldehyde and 4% sucrose in PBS for 15 minutes, washed three times with PBS, permeabilized with 0.3% Triton X-100 in PBS for 30 minutes, washed with PBS, and incubated for 1 hour at room temperature with blocking serum.

Cells and sections were incubated overnight or for 3 days, respectively, at 4°C with mouse monoclonal anti-DRD2 antibody (1:100), goat polyclonal anti-CHL1 antibody (1:100), and rabbit polyclonal anti-TB antibody or rabbit monoclonal anti-DARPP32 antibody (1:100 for cells, 1:200 for sections), all diluted in 0.5% carrageenan/PBS. The cells were washed with PBS, incubated for 1 hour at room temperature with Cy2-, Cy3-, and Cy5-conjugated secondary antibodies (1:200 in 0.5% carrageenan/PBS), washed with PBS, and mounted using mounting medium containing DAPI (100 µg/mL for cells, 500 µg/mL for sections) and Immu-Mount (Thermo Scientific). Images were taken at the confocal microscope (Olympus Fluoview FV1000, Olympus, Hamburg, Germany) in sequential mode with a 60× objective.

**2.10 Proximity ligation assay**

In proximity ligation, assay primary antibodies raised in different species and directed against two proteins and species-specific secondary antibodies labeled with different oligonucleotide probes are used. When the antigens are in close proximity (40 nm or less), the primary and secondary antibodies are also in close proximity, allowing the oligonucleotides of the secondary antibodies to hybridize to sequences of two circle-forming oligonucleotides. After ligation of the circle-forming oligonucleotides, DNA circles are amplified by PCR using circle-forming oligonucleotides as primers and the ligated circle as a template for the rolling-circle amplification. Fluorescently labeled oligonucleotide probes complementary to the repeated sequences of the rolling circles are added to bind to the amplified DNA and to visualize each close interaction as an individual fluorescent spot.

Here, the assay was performed on cultured cells after fixation or on fixed tissue sections after antigen retrieval using Duolink PLA reagents according to the manufacturer's protocol (Sigma-Aldrich; Duolink PLA technology). Briefly, tissue or cells were blocked with Duolink Blocking Solution, incubated overnight (cells) or for 3 days (tissue) with mouse monoclonal anti-DRD2 antibody (1:100) and goat polyclonal anti-CHL1 antibody (1:100) in Duolink Antibody Diluent, washed with Duolink Wash Buffer A, and incubated with a mixture of secondary antibodies conjugated with oligonucleotides (Duolink Anti-Mouse PLA Probe MINUS and Duolink Anti-Goat PLA Probe PLUS). Then the proximity ligation reaction, including hybridization/ligation of the circle-forming oligonucleotides, amplification of rolling circles, detection with fluorescently labeled oligonucleotides, and washing steps with Duolink Wash Buffer B were performed according to the manufacturer's protocol using the Duolink PLA Detection Reagent RED. Upon mounting with Duolink In Situ Mounting Medium with DAPI, the sections or cells were analyzed at the Olympus Fluoview FV1000 confocal laser-scanning microscope (Olympus) with a 60× objective. Quantification of red spots in confocal images was carried out using ImageJ.

For combining PLA and immunofluorescence, we slightly modified a previous protocol. Briefly, cells or tissue were blocked with Duolink Blocking Solution for 1 hour at 37°C, incubated overnight (cells) or for 3 days (tissue) at 4°C with mouse monoclonal anti-DRD2 antibody (1:50), goat polyclonal anti-CHL1 antibody (1:50), and rabbit polyclonal anti-TB antibody or rabbit monoclonal anti-DARPP32 antibody (1:100 for cells, 1:200 for slices) in Duolink Antibody Diluent. After washing with Duolink Wash Buffer A (2 × 5 minutes for cells, 3 × 5 minutes for sections), samples were incubated with Duolink Anti-Mouse PLA Probe MINUS and Duolink Anti-Goat PLA Probe PLUS for 1 hour at 37°C. Then, the proximity ligation reaction was performed according to the manufacturer’s protocol with slight modifications. For tissue sections, the dilutions of ligase and polymerase were modified to 1:30 and 1:60, respectively. Furthermore, ligation was performed for 45 minutes and polymerase reaction on slices for 150 minutes. Samples were washed for 10 minutes in Duolink Wash Buffer B and thereafter incubated with DAPI (100 µg/mL for cells, 500 µg/mL for sections) and Cy5-conjugated anti-rabbit secondary antibody (1:200) for 1 hour at room temperature. After 3 washes for 10 minutes each with Duolink Wash Buffer B, samples were mounted with Immu-Mount (Thermo Scientific) and analyzed with the Olympus Fluoview FV1000 confocal laser-scanning microscope (Olympus) using a 60× objective.

**2.11 Culture of ventral midbrain**

Ventral midbrain was removed from 14-day-old mouse embryos and cultures were prepared as described. Briefly, ventral midbrains were placed in ice-cold Dulbecco’s Modified Eagle Medium (DMEM) immediately after

---

**References**

58. Modified a previous protocol. Briefly, cells or tissue were blocked with Duolink Blocking Solution for 1 hour at 37°C, incubated overnight (cells) or for 3 days (tissue) at 4°C with mouse monoclonal anti-DRD2 antibody (1:50), goat polyclonal anti-CHL1 antibody (1:50), and rabbit polyclonal anti-TB antibody or rabbit monoclonal anti-DARPP32 antibody (1:100 for cells, 1:200 for slices) in Duolink Antibody Diluent. After washing with Duolink Wash Buffer A (2 × 5 minutes for cells, 3 × 5 minutes for sections), samples were incubated with Duolink Anti-Mouse PLA Probe MINUS and Duolink Anti-Goat PLA Probe PLUS for 1 hour at 37°C. Then, the proximity ligation reaction was performed according to the manufacturer’s protocol with slight modifications. For tissue sections, the dilutions of ligase and polymerase were modified to 1:30 and 1:60, respectively. Furthermore, ligation was performed for 45 minutes and polymerase reaction on slices for 150 minutes. Samples were washed for 10 minutes in Duolink Wash Buffer B and thereafter incubated with DAPI (100 µg/mL for cells, 500 µg/mL for sections) and Cy5-conjugated anti-rabbit secondary antibody (1:200) for 1 hour at room temperature. After 3 washes for 10 minutes each with Duolink Wash Buffer B, samples were mounted with Immu-Mount (Thermo Scientific) and analyzed with the Olympus Fluoview FV1000 confocal laser-scanning microscope (Olympus) using a 60× objective.

59. Briefly, ventral midbrains were placed in ice-cold Dulbecco’s Modified Eagle Medium (DMEM) immediately after
dissection and incubated in 5 ml fresh DMEM for 10 minutes at 37°C in a water bath. Afterwards, excess DMEM was discarded and ventral midbrains were mechanically dissociated with a 1 ml pipette tip in 5 ml pre-warmed primary culture medium, consisting of DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, 25 mM HEPES, 4 mM glutamine, and 30 mM glucose. The tissue suspension was allowed to settle for 2 minutes, and the supernatant was collected for counting cell numbers using trypan blue. Cells (1 × 10^5) were seeded at a density of 1 × 10^6 cell/mL onto glass coverslips coated with poly-D-lysine (0.1 mg/mL) and laminin (10 μg/mL) being placed in 12-multiwell plates, and incubated overnight in a humidified incubator with 5% CO_2/95% O_2 at 37°C. Then, 900 μL culture medium was carefully added to the wells and cells were further incubated. After 2 days, 50% of the cell culture supernatant was replaced by fresh culture medium and after 5 days, 50% of culture medium was replaced by DMEM-F12 medium supplemented with 1% N2 supplement and 10 μg/mL bovine serum albumin. Cultures were maintained for 2 further days before analysis.

2.12 | Cultures of striatum

Cultures of striatum from 16-day-old mouse embryos were prepared as described. Briefly, dissected striata were immediately transferred to ice-cold Hank's Balanced Salt Solution (HBSS), followed by centrifugation at 150 g for 5 minutes at room temperature. After removing excess HBSS, tissue was mechanically dissociated with a fire-polished glass pipette in 5 ml Primary Neuron Basal Medium (PNBM, Lonza, Cologne, Germany) containing 200 U/mL DNase. The suspension was allowed to settle for 2 minutes, and the supernatant was collected for counting cells using trypan blue. Cells were resuspended in PNB medium supplemented with PNFM SingleQuots (Lonza) and NFG-β (100 ng/mL) and 1 × 10^5 cells were seeded onto glass coverslips coated with poly-D-lysine (0.1 mg/mL) and laminin (10 μg/mL) being placed in 12-multiwell plates. Cultures were maintained in a humidified incubator with 5% CO_2/95% O_2 at 37°C for 14 days. Every 4 days, 50% of culture medium was replaced by freshly supplemented PNB.

2.13 | Statistical analysis

Values in graphs are presented as mean ± standard deviation, mean ± standard error of the mean, or as box plots. Data were analyzed by statistical tests as indicated in the legends. The threshold value for acceptance of differences between group mean values was 5%.

3 | RESULTS

3.1 | CHL1 directly interacts with the first extracellular loop of DRD2 via its extracellular domain

To investigate whether CHL1 and DRD2 interact, we performed immunoprecipitations with mouse brain homogenates and anti-CHL1, anti-NCAM and non-immune control antibodies followed by Western blot analysis of the immunoprecipitates with anti-DRD2 antibody. In the CHL1 and NCAM immunoprecipitates one immunopositive band of ~55 kDa, characteristic for DRD2, was detected, while only negligible amounts of protein with this apparent molecular mass were found in the immunoprecipitate of the control antibody (Figure 1A). Upon immunoprecipitations with anti-DRD2 and non-immune control antibodies, CHL1 was detected in the DRD2 immunoprecipitates by Western blot analysis at the expected molecular mass of ~200 kDa, while it was not detectable in the immunoprecipitates of the control antibody (Figure 1B). Using brain homogenates from CHL1+/+ and CHL1−/− mice for immunoprecipitation with anti-CHL1 antibody, DRD2 was detectable in the CHL1 immunoprecipitates from CHL1+/+, but not CHL1−/− brain homogenates (Figure 1C). These results indicate that CHL1 is associated with DRD2.

In a previous study, we have shown that the intracellular domain of the neural cell adhesion molecule NCAM interacts with DRD2, while the intracellular CHL1 domain which was used as control, showed no interaction with DRD2. Since an interaction of CHL1 with DRD2 via its intracellular domain has thus been excluded, we here tested whether the extracellular CHL1 domain mediates the interaction with DRD2. In this experiment, CHL1-Fc and NCAM-Fc were probed in a pull-down assay with mouse brain homogenate. Western blot analysis with anti-DRD2 antibody showed that CHL1-Fc, but not NCAM-Fc pulled down DRD2 from the brain homogenate (Figure 1D). This result indicates that CHL1 associates with DRD2 via its extracellular domain.

To analyze which the extracellular CHL1 and DRD2 domains bind to each other directly, ELISA experiments were performed. Synthetic peptides comprising the extracellular N-terminus or the first, second, or third extracellular loop of DRD2 were used as substrate-coat and incubated with different concentrations of CHL1-Fc or NCAM-Fc. CHL1-Fc, but not NCAM-Fc, showed a concentration-dependent and saturable binding to the first extracellular DRD2 loop, while both CHL1-Fc and NCAM-Fc showed neither binding to the DRD2 N-terminus nor to the second and third extracellular DRD2 loops (Figure 2A-D). These results show that CHL1 binds directly to the first extracellular loop of DRD2 via its extracellular domain.
To investigate whether the extracellular domain of CHL1 binds to DRD2 at the cell surface, HEK293 cells were transfected to express DRD2-S or DRD2-L, incubated with CHL1-Fc, and immunostained for DRD2 and CHL1. In DRD2-expressing cells, several CHL1-immunopositive signals were observed in areas of intense DRD2 immunostaining, indicating a partial overlap in CHL1 and DRD2 localization (Figure 3A,B). Neither CHL1-immunopositive
nor DRD2-immunopositive signals were observed in adjacent untransfected cells which do not express DRD2 (Figure 3A,B). This result indicates that CHL1 can interact via its extracellular domain with DRD2-S and DRD2-L in trans-orientation, signifying that CHL1 and DRD2 are present at the plasma membrane of different cells.

3.2 | CHL1 interacts with DRD2-S and DRD2-L in transfected HEK293 cells

To determine whether CHL1 interacts with DRD2-S and/or DRD2-L, proximity ligation assay was performed using HEK293 cells transfected to express DRD2-S or DRD2-L alone or together with CHL1, allowing specific detection of protein interactions in the range of 40 nm or less. Using anti-DRD2 and anti-CHL1 antibodies which both recognized extracellular epitopes, many DRD2/CHL1-positive fluorescent signals were observed as spots on cells co-expressing CHL1 and DRD2-S or DRD2-L, whereas no spots were observed on cells expressing only DRD2-S or DRD2-L (Figure 4A). This result indicates that CHL1 interacts with both DRD2 forms. Quantification of the DRD2/CHL1-positive spots showed approximately three times more spots positive for CHL1 and DRD2-S than spots positive for CHL1 and DRD2-L form (Figure 4B), suggesting that CHL1 predominantly interacts with DRD2-S. Phase contrast indicated that DRD2-S/CHL1-positive spots were predominantly located at the cell surface (Figure 4C), confirming the notion that DRD2-S and CHL1 interact at the cell surface via their extracellular domains.

3.3 | CHL1 reduces the agonist-induced internalization of DRD2-S

Quinpirole is an agonist of DRD2 and its binding to DRD2 triggers the internalization of DRD2. To analyze the effect of CHL1 on the agonist-induced internalization of DRD2-S and DRD2-L, we performed cell surface biotinylation of live cells after stimulation with or without quinpirole, isolated the biotinylated proteins from cell lysates using streptavidin beads and subjected cell lysates and biotinylated proteins to Western blot analysis with anti-DRD2 antibody. In the absence of CHL1 expression, cell surface levels of DRD2-S were decreased upon quinpirole application relative to those observed without quinpirole, while the levels were not reduced by quinpirole treatment in the presence of CHL1 (Figure 5A). The cell surface levels of DRD2-L were not altered by quinpirole in the presence and absence of CHL1 (Figure 5B), indicating that DRD2-L is not internalized upon quinpirole treatment independent of CHL1 expression. Expression and cell surface levels of CHL1 were not affected by quinpirole treatment (Figure 5C). Quantification of the cell surface levels and total levels in the cell lysates and comparison of cell surface levels normalized to total levels showed that the relative cell surface level of DRD2-S was reduced by approximately 40% by quinpirole in the absence of CHL1, whereas the level was not reduced by quinpirole in the presence of CHL1 (Figure 5D). The relative cell surface levels of DRD2-L in the absence and presence of CHL1 were not reduced by quinpirole (Figure 5D). These results indicate that CHL1 inhibits quinpirole-induced internalization of DRD2-S, but not of DRD2-L. Cell surface levels of DRD2-S and DRD2-L in the presence and absence of CHL1 were similar (Figure 5E). This result shows similar cell surface expression of DRD2-S and DRD2-L in the presence and absence of CHL1 and indicates that CHL1 does not affect cell surface expression of DRD2-S and DRD2-L.

The effect of CHL1 on agonist-induced internalization of DRD2-S was also analyzed by a different method, which
allows quantification of internalized DRD2-S. To this aim, transfected HEK293 cells expressing DRD2-S alone or together with CHL1 were incubated with anti-DRD2 antibody directed against the extracellular N-terminus. Unbound antibody was then removed and cells were stimulated with quinpirole to trigger internalization of receptor-bound antibody. After mild fixation, not allowing surface plasma membrane permeabilization, the cells were incubated with a Cy-3-conjugated secondary antibody to label non-internalized receptor-bound antibodies at the cell surface. After removal of unbound secondary antibodies, cells were permeabilized and incubated with Cy-2-conjugated secondary antibody to
Figure 5  Quinpirole reduces the cell surface level of DRD2-S in the absence of CHL1. A-C, HEK293 expressing DRD2-S (A) or DRD2-L (B) alone or co-expressing DRD2-S and CHL1 (DRD2-S/CHL1) (A, C) or DRD2-L and CHL1 (DRD2-L/CHL1) (B, C) were incubated with (+quin) or without (−quin) quinpirole followed by cell surface biotinylation, isolation of biotinylated proteins and Western blot analysis (WB) of the biotinylated proteins (surface) and the cell lysates (total) with anti-DRD2 (A, B) and anti-CHL1 (C) antibodies. The anti-GAPDH antibody was used to control loading (A, B). Total levels indicate the levels of CHL1 and DRD2 in cell lysates before isolation of biotinylated proteins, and cell surface levels represent biotinylated DRD2 after isolation of biotinylated proteins. A-C, Shown are representative blots from three independent experiments. D, E, Cell surface levels of DRD2-S and DRD2-L and total DRD2-S and DRD2-L levels in the cell lysates were determined, cell surface levels were normalized to total levels and the ratio of relative cell surface level after quinpirole treatment (+quin) and relative cell surface level after treatment without quinpirole (−quin) was calculated. Means ± standard deviation from three independent experiments are shown for the ratios of the cell surface levels with quinpirole treatment relative to the cell surface levels without quinpirole treatment (D) and for the cell surface levels without quinpirole treatment relative to the total levels without quinpirole treatment (E) (Kruskal-Wallis test with post-hoc Dunn’s multiple comparison test; **P < .01; ns: not significant)
To analyze whether the CHL1/DRD2 interaction takes place postsynaptically in DARPP32-expressing medium spiny neurons, which represent 90%-95% of the total neuronal population of the striatum, or whether this interaction occurs presynaptically in TH-expressing dopaminergic neurons, which innervate the medium spiny neurons in the striatum, or whether this interaction occurs presynaptically in TH-expressing dopaminergic neurons and postsynaptically on dopaminergic neurons and medium spiny neurons.

To substantiate this observation, cell cultures of striatum and ventral midbrain were analyzed by proximity ligation assay combined with immunofluorescent staining for TH or DARPP32. Interaction between CHL1 and DRD2 as seen by red spots was detected in TH-positive ventral midbrain cells (Figure 10A) and in DARPP32-positive striatal cells (Figure 10B), indicating that CHL1 and DRD2 interact postsynaptically in striatal medium spiny neurons and presynaptically in dopaminergic neurons innervating these neurons.

3.5 | DRD2 and pSer40-TH levels are reduced in the dorsal striatum of CHL1−/− mice, while pThr34-DARPP32 level is reduced in the ventral striatum of CHL1−/− mice

After agonist-induced internalization, DRD2 is targeted to late endosomes and lysosomes for degradation,
65 leading to reduced DRD2 levels. Since DRD2 interacts with CHL1 in the striatum and internalization of DRD2 is enhanced in the absence of CHL1, we investigated whether the enhanced internalization in the absence of CHL1 may be associated with a decreased level of DRD2 in CHL1−/− mice and alterations in DRD2-mediated pre- and/or postsynaptic signaling, for example, phosphorylation of TH at Ser40 (pSer40-TH) and of DARPP32 at Thr34 (pThr34-DARPP32), respectively. In the dorsal striatum of CHL1−/− mice, lower DRD2 and pSer40-TH levels were observed in comparison to those in CHL1+/+ mice (Figure 11A,B), whereas the levels of pThr34-DARPP32 were similar in CHL1+/+ and CHL1−/− mice (Figure 11C). In the ventral striatum, pThr34-DARPP32 levels were reduced in CHL1−/− mice (Figure 11C), while DRD2 and pSer40-TH levels were not altered relative to those in CHL1+/+ mice (Figure 11A,B). These results suggest that CHL1 is involved in regulation of pre- and postsynaptic signaling.

4 | DISCUSSION

Dopaminergic signaling through DRD2 is crucial for the function of the nervous system, and impairment in DRD2-mediated responses has been related to several neurological disorders.4,17-20 In search for mechanisms that may underlie these disorders, we identified CHL1 as a novel binding partner of DRD2 by several methods. CHL1 binds via its
extracellular domain to the first extracellular loop of DRD2, which is involved in the formation of an extended ligand binding pocket, being unique for DRD2 and mediating binding of antipsychotics, such as risperidone which is a DRD2 inverse agonist like most antipsychotics. In comparison to the first extracellular loops of other dopamine/aminergic receptors, the first extracellular loop of DRD2 adopts a unique conformation with a rotated structure exposing Trp100 to the top of the binding pocket. This crucial residue cooperates with Ile184 and Leu94 in the second extracellular loop and the first transmembrane domain which both contribute to the ligand binding. On the basis of these observations, it is tempting to speculate that binding of CHL1 to the first extracellular loop changes the conformation of DRD2.

We showed that CHL1 interacts with both DRD2-S and DRD2-L. DRD2-S is highly expressed in dopaminergic neurons and represents the main presynaptic autoreceptor in the dopaminergic system, yet not only regulating presynaptic autoreceptor activity but also neurotransmission postsynaptically. Studies using DRD2-L-deficient mice have indicated that DRD2-S acts predominantly as autoreceptor postsynaptically.

**FIGURE 6** Quinpirole-induced internalization of DRD2-S is reduced in the presence of CHL1. A, B, HEK293 expressing DRD2-S or co-expressing DRD2-S and CHL1 (DRD2-S/CHL1) were incubated with anti-DRD2 antibody against the extracellular N-terminus. After removal of unbound antibodies, cells were stimulated without (−quin) or with (+quin) quinpirole, fixed and incubated with Cy-3-conjugated secondary antibody. Cy-3-conjugated secondary antibodies (red) indicate non-internalized DRD2-bound anti-DRD2 antibodies at the cell surface and Cy-2-conjugated secondary antibodies (green) label internalized DRD2-bound anti-DRD2 antibodies. A, Representative images of HEK293 expressing DRD2-S after quinpirole treatment show surface DRD2 (red) and internalized DRD2 (green). Scale bar: 10 µm. B, Integrated densities of internalized and cell surface receptor-bound antibodies were determined and integrated densities of internalized receptor-bound antibodies were normalized to the total integrated densities (sum of integrated densities of internalized and cell surface receptor-bound antibodies). Box plots for the relative levels of internalized DRD2 are shown (**P < .01, ***P < .001; One-way ANOVA with post-hoc Student Newman-Keul’s test). The experiment was performed two times in duplicates.
while DRD2-L impinges dopaminergic transmission postsynaptically.\textsuperscript{72,76,77} When expressed in cultures of dopaminergic midbrain neurons, DRD2-S and DRD2-L are similar in subcellular localization and signaling.\textsuperscript{30} Similarly, virus-mediated expression of DRD2-S or DRD2-L in dopaminergic neurons from substantia nigra of DRD2-deficient mice

**FIGURE 7** Co-immunostaining of CHL1 and DRD2 in the striatum. Tissue sections from 12- to 18-week-old CHL1\textsuperscript{+/-} (A, C) and CHL1\textsuperscript{--/--} (B) mice were subjected to immunostaining using goat anti-CHL1 and mouse anti-DRD2 antibodies and Cy-3-conjugated anti-mouse and Cy-2-conjugated anti-goat secondary antibodies. Nuclei are stained with DAPI. Representative image of immunofluorescence staining for DAPI (blue), CHL1 (green), and DRD2 (red) are shown and yellow signals show co-localizations. C, Close-ups of two regions indicated by boxes in (A). A-C, Scale bars: 20 µm. Three independent experiments were performed with different sets of animals.
indicated that both isoforms are equally potent in acting as postsynaptic receptors and presynaptic autoreceptors. Based on these results, it has been proposed that the preferential autoreceptor function of DRD2-S is due to its predominant expression in dopaminergic neurons and not due to distinct subcellular localizations or signaling properties of DRD2-S and DRD2-L. It is of note in this context that CHL1 has been detected presynaptically to contribute to chaperoning synaptic vesicle exocytosis by regulating clathrin-dependent synaptic vesicle recycling. Moreover, not only DRD2 but also CHL1 are expressed in dopaminergic neurons. The findings of our study are in agreement with these observations: CHL1 and DRD2 co-localized in cultured TH-positive ventral midbrain neurons and in TH-positive cells in striatal sections, suggesting that CHL1 and presynaptic DRD2 autoreceptors interact presynaptically in cis-orientation at the presynaptic plasma membrane of dopaminergic terminals innervating medium spiny neurons. It is therefore conceivable that CHL1 regulates the autoreceptor functions of presynaptic DRD2-S, the predominant presynaptic DRD2 isoform.

We further show that CHL1 and DRD2 co-localize in cultured DARPP32-positive striatal cells and in DARPP32-positive cells in striatal sections, suggesting that CHL1 regulates postsynaptic DRD2 activity in striatal medium size spiny neurons, the predominant neuronal population of the striatum. Since we observed a trans-interaction of the soluble recombinant extracellular CHL1 domain with DRD2-S and DRD2-L at the surface of transfected HEK293 cells, it is conceivable that CHL1 at presynaptic or postsynaptic plasma membranes also interact in trans-orientation with DRD2 at opposite postsynaptic or presynaptic sites, thereby influencing pre- and postsynaptic DRD2 activities. Since CHL1 is also expressed by parvalbumin-immunopositive striatal GABAergic interneurons, it can be expected that cis- as well as trans-interactions between CHL1 and DRD2 are involved in DRD2-dependent regulation of GABAergic cell activities.

Using cell type-specific deletion of DRD2 in mice, it has been shown that postsynaptic DRD2 expressed by non-dopaminergic medium spiny neurons of the striatum participate in the inhibitory feedback control of presynaptic D2 function, for example, dopamine synthesis and release. This effect is more pronounced in the dorsal striatum than in the ventral striatum, indicating that the regulation of dopamine synthesis and release is not solely regulated by presynaptic DRD2 autoreceptors on dopaminergic neurons, but that postsynaptic DRD2 can participate in these functions, indicating that mesolimbic- and nigrostriatal-mediated functions are differentially regulated by postsynaptic DRD2.

In the present study, we show that CHL1 reduces the agonist-induced internalization of DRD2-S and that DRD2 levels are reduced in the dorsal striatum of CHL1−/− mice. These results suggest that internalization and degradation of autoreceptors in the dorsal striatum is enhanced in the absence of CHL1. Since it has been shown that DRD2 autoreceptors on dopaminergic neurons are resistant to agonist-induced internalization, we propose that in CHL1+/+ mice and, thus in the presence of CHL1, autoreceptors are not internalized. Since we did not detect agonist-induced internalization of DRD2-L in transfected HEK293 cells, we could not address the question whether CHL1 also affects the internalization of pre- and/or postsynaptic DRD2-L.

In CHL1−/− mice, we observed reduced pSer40-TH levels in the dorsal striatum and reduced pThr34-DARPP32

**FIGURE 8** Co-localization of CHL1 and DRD2 in the striatum. Tissue sections from 12- to 18-week-old CHL1+/+ and CHL1−/− mice were analyzed by proximity ligation assay using goat anti-CHL1 and mouse anti-DRD2 antibodies. Nuclei are stained with DAPI (blue). Representative images are shown at low (left and middle panel) and high (right panel) magnifications. Red spots indicate close molecular interaction of CHL1 with DRD2 in CHL1+/+ mice. CHL1−/− mice served as controls. Scale bars: 10 µm. Three independent experiments were performed with different sets of animals.
levels in the ventral striatum. These findings suggest that CHL1 affects presynaptic activity in the dorsal striatum and postsynaptic activity in the ventral striatum. Reduced pSer40-TH and pThr34-DARPP32 levels could result from inhibition of DARPP32 and TH phosphorylation.\textsuperscript{71}77 Since we did not find enhanced DRD2 levels in the ventral striatum of CHL1\textsuperscript{−/−} mice and observed reduced rather than enhanced levels in the dorsal striatum of CHL1\textsuperscript{−/−} mice, the reduced pSer40-TH or pThr34-DARPP32 levels in the dorsal or ventral striatum of CHL1\textsuperscript{−/−} mice are not due to the reduced levels of pre- and postsynaptic DRD2. It is therefore likely that the reduced activity levels are caused by alterations in

**FIGURE 9** Interaction of CHL1 and DRD2 on TH- and DARPP32-positive neurons in striatal sections. Proximity ligation assay using goat anti-CHL1 and mouse anti-DRD2 antibodies was combined with immunostaining using rabbit anti-DARPP32 (A) or anti-TH (B) antibodies to analyze tissue sections from 12- to 18-week-old CHL1\textsuperscript{+/+} mice. Nuclei are stained with DAPI (blue). Representative images are shown. Close-ups of two regions (without DAPI staining) are indicated by boxes and arrowheads indicate red spots indicating close molecular interaction of CHL1 with DRD2. Scale bars: 10 µm. Three independent experiments were performed with different sets of animals.
the activity of pre- and postsynaptic DRD2, since DRD2 can exist in functionally different receptor conformations, such as interconvertible high- and low-affinity states and constitutively active conformations. In addition, inverse agonism for DRD2 implies that this receptor can adopt a constitutively active conformation. A constitutively active conformation of DRD2 has been observed in several DRD2 mutants, in DRD2 chimera with other receptors and in a DRD2-S expressing cell line. These observations suggest that under certain conditions constitutively active DRD2 exists in vivo. We propose that presynaptic DRD2 is constitutively active in the dorsal striatum in the absence of CHL1 leading to a reduced Ser40 phosphorylation and activity of TH and thus to a reduced dopamine synthesis and release. It is also conceivable that the lack of CHL1 leads to an altered conformation of postsynaptic DRD2 and to an altered downstream signaling and reduced Thr34 phosphorylation of DARPP32 in the ventral striatum and thus to an altered postsynaptic activity.

Since DRD2 is internalized in its low-affinity state upon dopamine stimulation, we would like to speculate that some presynaptic autoreceptors in CHL1−/− mice are converted to the low-affinity state, being internalized and degraded, and thus leading to a reduced DRD2 level in the dorsal striatum of CHL1−/− mice. It is also conceivable that CHL1 modulates the interaction of presynaptic DRD2 with components of the endocytic machinery to prevent internalization of DRD2, thereby affecting the interaction of DRD2 with G-proteins.
and/or other signal transduction molecules to alter the pre-synaptic DRD2-dependent signaling pathways. These views call for analyzing the mechanisms underlying the regulation of DRD2 endocytosis and DRD2-dependent signaling by CHL1.

Our observations provide evidence that CHL1 modulates presynaptic and postsynaptic DRD2-dependent functions of the dopaminergic system. Since DRD2 and CHL1 are implicated in mental disorders, future investigations of the mechanism and the functional consequences of the CHL1-dependent
regulation of the dopaminergic system should provide new insights into therapeutic possibilities.

ACKNOWLEDGMENTS
M. Schachner is grateful to the Li Kashing Foundation for support.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
R. Kleene and M. Schachner designed the research; A. Kotarska and L. Fernandes performed the research; A. Kotarska, L. Fernandes, and R. Kleene analyzed the data; R. Kleene, A. Kotarska, and M. Schachner wrote the paper.

REFERENCES
1. Kobayashi M, Iaccarino C, Saiardi A, et al. Simultaneous absence of dopamine D1 and D2 receptor-mediated signaling is lethal in mice. Proc Natl Acad Sci U S A. 2004;101:11465-11470.
2. Klein MO, Battagello DS, Cardoso AR, Hauser DN, Bittencourt JC, Correa RG. Dopamine: Functions, signaling, and association with neurological diseases. Cell Mol Neurobiol. 2019;39:31-59.
3. Chong TT, Husain M. The role of dopamine in the pathophysiology and treatment of apathy. Prog Brain Res. 2016;229:389-426.
4. Iversen SD, Iversen LL. Dopamine: 50 years in perspective. Trends Neurosci. 2007;30:188-193.
5. Zhou QY, Palmiter RD. Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. Cell. 1995;83:1197-1209.
6. Sierra M, Carnicella S, Strafella AP, et al. Apathy and impulse control disorders: Yin & Yang of dopamine dependent behaviors. J Parkinsons Dis. 2015;5:625-636.
7. Arias-Carrion O, Poppel E. Dopamine, learning, and reward-seeking behavior. Acta Neurobiol Exp (Wars). 2007;67:481-488.
8. Arreola R, Alvarez-Herrera S, Perez-Sanchez G, et al. Immunomodulatory effects mediated by dopamine. J Immunol Res. 2016;2016:3160486.
9. Salamone JD, Pardo M, Yohn SE, Lopez-Cruz L, San Miguel N, Correa M. Mesolimbic dopamine and the regulation of motivated behavior. Curr Top Behav Neurosci. 2016;27:231-257.
10. Sarkar C, Basu B, Chakroverty D, Dasgupta PS, Basu S. The immunoregulatory role of dopamine: an update. Brain Behav Immun. 2010;24:525-528.
11. Belujon P, Grace AA. Dopamine system dysregulation in major depressive disorders. Int J Neuropsychopharmacol. 2017;20:1036-1046.
12. Beaulieu JM, Gainetdinov RR. The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol Rev. 2011;63:182-217.
13. Goto Y, Grace AA. The dopamine system and the pathophysiology of schizophrenia: a basic science perspective. Int Rev Neurobiol. 2007;84:61-88.
14. Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev. 1998;78:189-225.
15. Seeman P, Van Tol HH. Dopamine receptor pharmacology. Trends Pharmacol Sci. 1994;15:264-270.
16. Mailman RB, Huang X. Dopamine receptor pharmacology. Handb Clin Neurol. 2007;83:77-105.
17. Wong DF, Wagner HN Jr, Tune LE, et al. Positron emission tomography reveals elevated D2 dopamine receptors in drug-naive schizophrenics. Science. 1986;234:1558-1563.
18. Abi-Dargham A, Rodenhiser J, Pritzl D, et al. Increased baseline occupancy of D2 receptors by dopamine in schizophrenia. Proc Natl Acad Sci U S A. 2000;97:8104-8109.
19. Weinstein JJ, van de Giessen E, Rosengard RJ, et al. PET imaging of dopamine-D2 receptor internalization in schizophrenia. Mol Psychiatry. 2018;23:1506-1511.
20. Seeman P. Targeting the dopamine D2 receptor in schizophrenia. Expert Opin Ther Targets. 2006;10:515-531.
21. David C, Ewert M, Seeburg PH, Fuchs S. Antipeptide antibodies differentiate between long and short isoforms of the D2 dopamine receptor. Biochem Biophys Res Commun. 1991;179:824-829.
22. Fishburn CS, Elazar Z, Fuchs S. Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. J Biol Chem. 1995;270:29819-29824.
23. Dal Toso R, Sommer B, Ewert M, et al. The dopamine D2 receptor: two molecular forms generated by alternative splicing. EMBO J. 1989;8:4025-4034.
24. L’Hirondel M, Cheramy A, Godeheu G, et al. Lack of autoreceptor-mediated inhibitory control of dopamine release in striatal synaptosomes of D2 receptor-deficient mice. Brain Res. 1998;792:253-262.
25. Mercuri NB, Saiardi A, Bonci A, et al. Loss of autoreceptor function in dopaminergic neurons from dopamine D2 receptor deficient mice. Neuroscience. 1997;79:323-327.
26. Anzalone A, Lizardi-Ortiz JE, Ramos M, et al. Dual control of dopamine synthesis and release by presynaptic and postsynaptic dopamine D2 receptors. J Neurosci. 2012;32:9023-9034.
27. De Mei C, Ramos M, Itaka C, Borrelli E. Getting specialized: presynaptic and postsynaptic dopamine D2 receptors. Curr Opin Pharmacol. 2009;9:53-58.
28. Benoit-Marand M, Borrelli E, Gonnin F. Inhibition of dopamine release via presynaptic D2 receptors: time course and functional characteristics in vivo. J Neurosci. 2001;21:9134-9141.
29. Neve KA, Ford CP, Buck DC, Grandy DK, Neve RL, Phillips TJ. Normalizing dopamine D2 receptor-mediated responses in D2 null mutant mice by virus-mediated receptor restoration: comparing D2L and D2S. Neuroscience. 2013;248:479-487.
30. Jomphe C, Tiberi M, Trudeau LE. Expression of D2 receptor isoforms in cultured neurons reveals equipotent autoreceptor function. Neuropharmacology. 2006;50:595-605.
31. Menegus SE, Heimert TL, Odife ER, Quasney MW. A region of the third intracellular loop of the short form of the D2 dopamine receptor dictates Gi coupling specificity. J Biol Chem. 2004;279:1601-1606.
32. Leysen JE, Gommeren W, Mertens J, et al. Comparison of in vitro binding properties of a series of dopamine antagonists and agonists for cloned human dopamine D2S and D2L receptors and for D2 receptors in rat striatal and mesolimbic tissues, using [125I] 2'-iodosipiperone. Psychopharmacology. 1993;110:27-36.
33. Castro SW, Strange PG. Differences in the ligand binding properties of the short and long versions of the D2 dopamine receptor. J Neurochem. 1997;60:372-375.
34. Holm J, Hillenbrand R, Steuber V, et al. Structural features of a close homologue of L1 (CHL1) in the mouse: a new member of
the L1 family of neural recognition molecules. *Eur J Neurosci.* 1996;8:1613-1629.
35. Angeloni D, Lindor NM, Pack S, Latif F, Wei MH, Lerman MI. CALL gene is haploinsufficient in a 3p- syndrome patient. *Am J Med Genet.* 1999;86:482-485.
36. Angeloni D, Wei MH, Lerman MI. Two single nucleotide polymorphisms (SNPs) in the CALL gene for association studies with IQ. *Psychiatr Genet.* 1999;9:165-167.
37. Sakurai K, Migita O, Toru M, Arinami T. An association between a missense polymorphism in the close homologue of L1 (CHL1, CALL) gene and schizophrenia. *Mol Psychiatry.* 2002;7:421-415.
38. Frints SG, Marynen P, Hartmann D, et al. CALL interrupted in a patient with non-specific mental retardation: gene dosage-dependent alteration of murine brain development and behavior. *Hum Mol Genet.* 2003;12:1463-1474.
39. Chen QY, Chen Q, Feng GY, et al. Case-control association study of the close homologue of L1 (CHL1) gene and schizophrenia in the Chinese population. *Schizophr Res.* 2005;73:269-274.
40. Chu TT, Liu Y. An integrated genomic analysis of gene-function correlation on schizophrenia susceptibility genes. *J Hum Genet.* 2010;55:285-292.
41. Tam GW, van de Lagemaat LN, Redon R, et al. Confirmed rare copy number variants implicate novel genes in schizophrenia. *Biochim Biophys Acta.* 2010;180:445-451.
42. Cuocò C, Ronchetto P, Gimelli S, et al. Microarray based analysis of an inherited terminal 3p26.3 deletion, containing only the CHL1 gene, from a normal father to his two affected children. *Orphanet J Rare Dis.* 2011;6:12.
43. Salyakina D, Cukier HN, Lee JM, et al. Copy number variants in extended autism spectrum disorder families reveal candidates potentially involved in autism risk. *PLoS ONE.* 2011;6:e26049.
44. Shoukier M, Fuchs S, Schwabold E, et al. Microduplication of 3p26.3 in nonsyndromic intellectual disability indicates an important role of CHL1 for normal cognitive function. *Neuropediatrics.* 2013;44:268-271.
45. Irinchev A, Koch M, Needham LK, Maness P, Schachner M. Impairment of sensorimotor gating in mice deficient in the cell adhesion molecule L1 or its close homologue, CHL1. *Brain Res.* 2004;1029:131-134.
46. Nikonenko AG, Sun M, Lepsveridze E, et al. Enhanced perisomatic inhibition and impaired long-term potentiation in the CA1 region of juvenile CHL1-deficient mice. *Eur J Neurosci.* 2006;23:1839-1852.
47. Montag-Sallaz M, Baarke A, Montag D. Aberrant neuronal connectivity in CHL1-deficient mice is associated with altered information processing-related immediate early gene expression. *J Neurobiol.* 2003;57:67-80.
48. Montag-Sallaz M, Schachner M, Montag D. Misguided axonal projections, neural cell adhesion molecule 180 mRNA upregulation, and altered behavior in mice deficient for the close homolog of L1. *Mol Cell Biol.* 2002;22:7967-7981.
49. Pratte M, Jamon M. Impairment of novelty detection in mice targeted for the Chl1 gene. *Physiol Behav.* 2009;97:394-400.
50. Pratte M, Rougon G, Schachner M, Jamon M. Mice deficient for the close homologue of the neural adhesion cell L1 (CHL1) display alterations in emotional reactivity and motor coordination. *Behav Brain Res.* 2003;147:31-39.
51. Morellini F, Lepsveridze E, Kahler B, Dityatev A, Schachner M. Reduced reactivity to novelty, impaired social behavior, and enhanced basal synaptic excitatory activity in perforant path projections to the dentate gyrus in young adult mice deficient in the neural cell adhesion molecule CHL1. *Mol Cell Neurosci.* 2007;34:121-136.
52. Morellini F, Sivukhina E, Stoenica L, et al. Improved reversal learning and working memory and enhanced reactivity to novelty in mice with enhanced GABAergic innervation in the dentate gyrus. *Cereb Cortex.* 2010;20:2712-2727.
53. Maness PF, Schachner M. Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. *Nat Neurosci.* 2007;10:19-26.
54. Sytnyk V, Leshchyn'ska I, Schachner M. Neuronal cell adhesion molecules of the immunoglobulin superfamily regulate synaptic formation, maintenance, and function. *Trends Neurosci.* 2017;40:295-308.
55. Rolf B, Lang D, Hillenbrand R, Richter M, Schachner M, Bartsch U. Altered expression of CHL1 by glial cells in response to optic nerve injury and intravitreal application of fibroblast growth factor-2. *J Neurosci Res.* 2003;71:835-843.
56. Niethammer P, Delling M, Sytnyk V, Dityatev A, Fukami K, Schachner M. Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis. *J Cell Biol.* 2002;157:521-532.
57. Shen S, Mantei N, Dong L, Schachner M. Prevention of neuronal cell death by neural adhesion molecules L1 and CHL1. *J Neurobiol.* 1999;38:428-439.
58. Gomes I, Sierra S, Devi LA. Detection of receptor heteromerization using in situ proximity ligation assay. *Curr Protoc Pharmacol.* 2016;75, 2.16.1-2.16.31.
59. Choi WS, Kim HW, Xia Z. Preparation of primary cultured dopaminergic neurons from mouse brain. *Methods Mol Biol.* 2013;1018:61-69.
60. Schock SC, Jolin-Dahel KS, Schock PC, Staines WA, Garcia-Munoz M, Arbuthnott GW. Striatal interneurons in dissociated cell culture. *Histochim Cell Biol.* 2010;134:1-12.
61. Xiao MF, Xu JC, Tereshchenko Y, Novak D, Schachner M, Kleene R. Neural cell adhesion molecule modulates dopaminergic signaling and behavior by regulating dopamine D2 receptor internalization. *J Neurosci.* 2009;29:14752-14763.
62. Guo N, Guo W, Kralikova M, et al. Impact of D2 receptor internalization on binding affinity of neuroimaging radiotracers. *Neuropsychopharmacology.* 2010;35:806-817.
63. Sander CY, Hooker JM, Catana C, Rosen BR, Mandeville JB. Imaging agonist-induced D2/D3 receptor desensitization and internalization in vivo with PET/fMRI. *Neuropsychopharmacology.* 2016;41:1427-1436.
64. Kessler RM, Woodward ND, Riccardi P, et al. Dopamine D2 receptor levels in striatum, thalamus, substantia nigra, limbic regions, and cortex in schizophrenic subjects. *Biol Psychiatry.* 2009;65:1024-1031.
65. Bartlett SE, Enquist J, Hopf FW, et al. Dopamine responsiveness is regulated by targeted sorting of D2 receptors. *Proc Natl Acad Sci U S A.* 2005;102:11521-11526.
66. Wang S, Che T, Levit A, Shoichet BK, Wacker D, Roth BL. Structure of the D2 dopamine receptor bound to the atypical antipsychotic drug risperidone. *Nature.* 2018;555:269-273.
67. Roberts DJ, Lin H, Strange PG. Mechanisms of agonist action at D2 dopamine receptors. *Mol Pharmacol.* 2004;66:1573-1579.
68. Roberts DJ, Strange PG. Mechanisms of inverse agonist action at D2 dopamine receptors. *Br J Pharmacol.* 2005;145:34-42.
Shi L, Javitch JA. The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice. *Proc Natl Acad Sci U S A*. 2004;101:440-445.

Shi L, Simpson MM, Ballesteros JA, Javitch JA. The first transmembrane segment of the dopamine D2 receptor: accessibility in the binding-site crevice and position in the transmembrane bundle. *Biochemistry*. 2001;40:12339-12348.

Khan ZU, Mrzljak L, Gutierrez A, de la Calle A, Goldman-Rakic PS. Prominence of the dopamine D2 short isoform in dopaminergic pathways. *Proc Natl Acad Sci U S A*. 1998;95:7731-7736.

Usiello A, Baik JH, Rouge-Pont F, et al. Distinct functions of the two isoforms of dopamine D2 receptors. *Nature*. 2000;408:199-203.

Joseph JD, Wang YM, Miles PR, et al. Dopamine autoreceptor regulation of release and uptake in mouse brain slices in the absence of D(3) receptors. *Neuroscience*. 2002;112:39-49.

Rouge-Pont F, Usiello A, Benoît-Marand M, Gonon F, Piazza PV, Borrelli E. Changes in extracellular dopamine induced by morphine and cocaine: crucial control by D2 receptors. *J Neurosci*. 2002;22:3293-3301.

Centonze D, Usiello A, Gubellini P, et al. Dopamine D2 receptor-mediated inhibition of dopaminergic neurons in mice lacking D2L receptors. *Neuropsychopharmacology*. 2002;27:723-726.

Wang Y, Xu R, Sasaoka T, Tonegawa S, Kung MP, Sankoorikal EB. Dopamine D2 long receptor-deficient mice display alterations in striatum-dependent functions. *J Neurosci*. 2000;20:8305-8314.

Lindgren N, Usiello A, Goiny M, et al. Distinct roles of dopamine D2L and D2S receptor isoforms in the regulation of protein phosphorylation at presynaptic and postsynaptic sites. *Proc Natl Acad Sci U S A*. 2003;100:4305-4309.

Andreyeva A, Leshchyns’ka I, Knepper M, et al. CHL1 is a selective organizer of the presynaptic machinery chaperoning the SNARE complex. *PLoS ONE*. 2010;5:e12018.

Leshchyns’ka I, Sytnyk V, Richter M, Andreyeva A, Puchkov D, Schachner M. The adhesion molecule CHL1 regulates uncoating of clathrin-coated synaptic vesicles. *Neuron*. 2006;52:1011-1025.

Alsanie WF, Penna V, Schachner M, Thompson LH, Parish CL. Homophilic binding of the neural cell adhesion molecule CHL1 regulates development of ventral midbrain dopaminergic pathways. *Sci Rep*. 2017;7:9368.

Bye CR, Jonsson ME, Bjorklund A, Parish CL, Thompson LH. Transcriptome analysis reveals transmembrane targets on transplantable midbrain dopamine progenitors. *Proc Natl Acad Sci U S A*. 2015;112:E1946-E1955.

Kleene R, Chaudhary H, Karl N, et al. Interaction between CHL1 and serotonin receptor 2c regulates signal transduction and behavior in mice. *J Cell Sci*. 2015;128:4642-4652.

Robinson BG, Bunzow JR, Grimm JB, et al. Desensitized D2 auto-receptors are resistant to trafficking. *Sci Rep*. 2017;7:4379.

Skinbjerg M, Sibley DR, Javitch JA, Abi-Dargham A. Imaging the high-affinity state of the dopamine D2 receptor in vivo: fact or fiction? *Biochem Pharmacol*. 2012;83:193-198.

Sibley DR, De Lean A, Creese I. Anterior pituitary dopamine receptors. Demonstration of interconvertible high and low affinity states of the D-2 dopamine receptor. *J Biol Chem*. 1982;257:6351-6361.

Wreggett KA, Seeman P. Agonist high- and low-affinity states of the D2-dopamine receptor in calf brain. Partial conversion by guanine nucleotide. *Mol Pharmacol*. 1984;25:10-17.

Zhang B, Albaker A, Plouffe B, Lefebvre C, Tiberi M. Constitutive activities and inverse agonism in dopamine receptors. *Adv Pharmacol*. 2014;70:175-214.

Roberts DJ, Lin H, Strange PG. Investigation of the mechanism of agonist and inverse agonist action at D2 dopamine receptors. *Biochem Pharmacol*. 2004;67:1657-1665.

Hall DA, Strange PG. Evidence that antipsychotic drugs are inverse agonists at D2 dopamine receptors. *Br J Pharmacol*. 1997;121:731-736.

Strange PG. Agonism and inverse agonism at dopamine D2-like receptors. *Clin Exp Pharmacol Physiol Suppl*. 1999;26:S3-S9.

Kozell LB, Neve KA. Constitutive activity of a chimeric D2/D1 dopamine receptor. *Mol Pharmacol*. 1997;52:1137-1149.

Bullock CM, Li C, Li M, Bermak JC, Zhou QY. Sensitization of adenylylate cyclase induced by a dopamine D2 receptor mutant: inverse agonism by D2 receptor antagonists. *Prog Neuropsychopharmacol Biol Psychiatry*. 2001;25:1387-1402.

Ko F, Seeman P, Sun WS, Kapur S. Dopamine D2 receptors internalize in their low-affinity state. *NeuroReport*. 2002;13:1017-1020.

---

**How to cite this article:** Kotarska A, Fernandes L, Kleene R, Schachner M. Cell adhesion molecule close homolog of L1 binds to the dopamine receptor D2 and inhibits the internalization of its short isoform. *The FASEB Journal*. 2020;34:4832–4851. [https://doi.org/10.1096/fj.201900577RRRR](https://doi.org/10.1096/fj.201900577RRRR)