Identification of a small-molecule inhibitor of the PICK1 PDZ domain that inhibits hippocampal LTP and LTD

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Proteins containing PSD-95/Discs-large/ZO-1 homology (PDZ) domains play key roles in the assembly and regulation of cellular signaling pathways and represent putative targets for new pharmacotherapeutics. Here we describe the first small-molecule inhibitor (FSC231) of the PDZ domain in protein interacting with C kinase 1 (PICK1) identified by a screening of ~44,000 compounds in a fluorescent polarization assay. The inhibitor bound the PICK1 PDZ domain with an affinity similar to that observed for endogenous peptide ligands (K_i~10.1 μM). Mutational analysis, together with computational docking of the compound in simulations starting from the PDZ domain structure, identified the binding mode of FSC231. The specificity of FSC231 for the PICK1 PDZ domain was supported by the lack of binding to PDZ domains of postsynaptic density protein 95 (PSD-95) and glutamate receptor interacting protein 1 (GRIP1). Pretreatment of cultured hippocampal neurons with FSC231 inhibited coimmunopreciptation of the AMPA receptor GluR2 subunit with PICK1. In agreement with inhibiting the role of PICK1 in GluR2 trafficking, FSC231 accelerated recycling of pHluorin-tagged GluR2 in hippocampal neurons after internalization in response to NMDA receptor activation. FSC231 blocked the expression of both long-term depression and long-term potentiation in hippocampal CA1 neurons from acute slices, consistent with inhibition of the bidirectional function of PICK1 in synaptic plasticity. Given the proposed role of the PICK1/AMPA receptor interaction in neuropathic pain, excitotoxicity, and cocaine addiction, FSC231 might serve as a lead in the future development of new therapeutics against these conditions.

drug discovery | fluorescence polarization | protein–protein interactions | synaptic plasticity | AMPA receptors

The majority of currently available pharmacotherapeutics are targeted toward transmembrane receptor proteins such as G-protein-coupled receptors (1). This is not surprising, given the impressive functional diversity of these proteins that allows for selective inhibition or activation of distinct disease-modulating signaling pathways. Nevertheless, receptor activation or blockade will inevitably affect the entire ensemble of signaling pathways to which the receptor is coupled and thereby often cause not only beneficial effects but also unwanted side effects (2, 3). An attractive alternative would be to develop medicines that instead target protein–protein interactions in a specific intracellular signal-transduction pathway (4, 5).

PSD-95/Discs-large/ZO-1 homology (PDZ) domains seem well suited for such efforts because they have a confined groove that typically binds the C-terminal three to four residues of the interaction partner (6) and thereby also are likely to accommodate nonpeptide small-molecule inhibitors (7). Additionally, PDZ domains are among the most common protein domains in the human genome serving important roles in protein trafficking as well as in the formation of multiprotein signaling complexes (6, 8). Prototypical scaffolding proteins include postsynaptic density protein 95 (PSD-95) and glutamate receptor interacting protein 1 (GRIP1) that contain several PDZ domains and operate as molecular adapters in neuronal synapses (6, 8).

Recent findings support the idea that PDZ domains might indeed be valuable drug targets. Blocking the PDZ interaction between the NMDA glutamate receptor and PSD-95 with membrane-permeable peptides results in selective inhibition of neuronal nitric oxide synthase (nNOS) activation, which is expected to reduce ischemic brain injury during stroke (2, 3). In cancer, recent evidence suggests that blocking the PDZ domains of Na^+/H^+ exchanger regulatory factor 1 (NHERF-1), dishevelled, or AF-6 might be interesting therapeutic approaches (9–11). Furthermore, the PDZ domain of protein interacting with C kinase 1 (PICK1), which, e.g., binds the C terminus of AMPA-type ionotropic glutamate receptors (AMPA receptors) (12), has recently been recognized as a putative target in the treatment of neuropathic pain (13), excitotoxicity (14), and cocaine addiction (15).

Efforts have consequently been directed toward identification of small-molecule nonpeptide PDZ domain inhibitors that could serve as leads in future drug discovery efforts (6, 7). However, only a few compounds have been identified, and in general they display low affinities for their target (>100 μM) (10, 11, 16–18). Here we report the identification of a nonpeptide small-molecule inhibitor (FSC231) of the PICK1 PDZ domain. The compound has an affinity similar to that observed for the endogenous peptide ligands (K_i ~10 μM) and displays highly interesting pharmacological activity, as demonstrated by its ability to affect AMPA receptor trafficking and to inhibit synaptic plasticity in hippocampal CA1 neurons.

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Conflict of interest statement: T.S.T., K.L.M., T.D., D.P., L.C.B.R., and U.G. have filed a patent application for FSC231.

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Results

Identification of FSC231 as a Small-Molecule Inhibitor of the PICK1 PDZ Domain. To identify small-molecule inhibitors of the PICK1 PDZ domain, we used a fluorescence polarization (FP) assay that detects binding of fluorescently labeled peptides to the PDZ domain of purified PICK1 in solution (19). We used a 96-well format and an Oregon Green-labeled peptide (OG-DAT C13) corresponding to the 13 C-terminal residues of the dopamine transporter (DAT), a potent ligand of the PICK1 PDZ domain (19), to screen a part of the small-molecule screening collection at Neurosearch A/S (total number of screened compounds 43,880) for their ability to compete for binding of the fluorescent peptide. More than a hundred potentially interacting compounds were identified (defined by a >20% reduction in FP signal); however, subsequent validation reduced the number of confirmed hits to <15. One compound, FSC231 [(E)-ethyl 2-cyano-3(3,4-dichlorophenyl)acryloylcarbamate] (Fig. 1*A*), was chosen for further characterization. Competition FP assays showed potent dose-dependent inhibition of OG-DAT C13 binding to PICK1 [K_i = 10.1 μM (8.9; 11.3 μM), mean (SE interval), n = 9] (Fig. 1*B*). A closely related analog of FSC231 without the cyano group (FSC231_9) (Fig. 1*A*) did not inhibit OG-DAT C13 binding (K_i > 1000 μM) (Fig. 1*B*). At the excitation/emission wavelengths used for detection of Oregon Green fluorescence, we could not detect autofluorescence from FSC231 in concentrations up to 1 mM. To exclude that spectral properties of FSC231 interfered with the FP assay, we used, in addition, a DAT C13 peptide-labeled with Cy5 (Cy5-DAT C13). FSC231 also potently inhibited binding of this peptide (K_i ~10 μM, mean of n = 2).

We confirmed the ability of FSC231 to block the interaction between PICK1 and DAT using a method not based on fluorescence. In agreement with previous data (20), the C terminus of DAT (24 C-terminal residues) fused to gluthathione-*S*-transferase (GST) pulled down purified PICK1 (Fig. 1*C*). The pull-down was blocked by a C-terminal peptide of the Glt1b glutamate transporter (10 residues) (Fig. 1*C*), the most potent ligand for the PICK1 PDZ domain identified so far (21). The pull-down was also blocked in a dose-dependent manner by FCS231, but only marginally by FSC231_9 (Fig. 1*C*). We further demonstrated that FSC231 most likely is a reversible inhibitor (*SI Note 1*).

Binding Mode Prediction of FSC231 in the PDZ-Binding Crevice. FSC231 was docked into the crystallographic structure of the PDZ domain of human PICK1 (22) and into a mutant of the PDZ domain where Lys83 was substituted with histidine (K83H), known from our previous studies to affect the peptide-binding specificity (19). The position of the compound in the ligand-binding cleft was refined and stabilized with Molecular Dynamics (MD) simulations. The ligand explored two orientations in the PDZ-binding crevice in which it is flipped 180° with respect to the other (*SI Note 2*). In one mode (mode 1), which was preferred in the MD mulations for both wild type and K83H, the phenyl ring of FSC231 interacts at the P_0 position (the position corresponding to the C-terminal residue of the peptide ligand) (Fig. 1*D* and Fig. S1). Of interest, the mutation produced according to the MD simulations a local change in the shape of the binding pocket that was sufficient to establish a clear preference for the phenyl ring at P_0. Calculations of the free energy of binding (*SI Methods*) also showed a ΔΔG ~4 kcal/mol in favor of the mutant complex. This agrees with FP binding experiments showing a higher affinity of FSC231 for K83H than for the wild type [K83H, K_i = 2.2 μM (1.8; 2.1), mean (SE interval), n = 3] (Fig. S2).

FSC231 Binds with an Affinity Similar to the Natural Peptide Ligands and Shows Specificity for the PICK1 PDZ Domain. The binding of the AMPA receptor GluR2 C terminus to the PICK1 PDZ domain is the most well-studied interaction of PICK1 and is believed to play a key role in long-term depression (LTD) as well as in long-

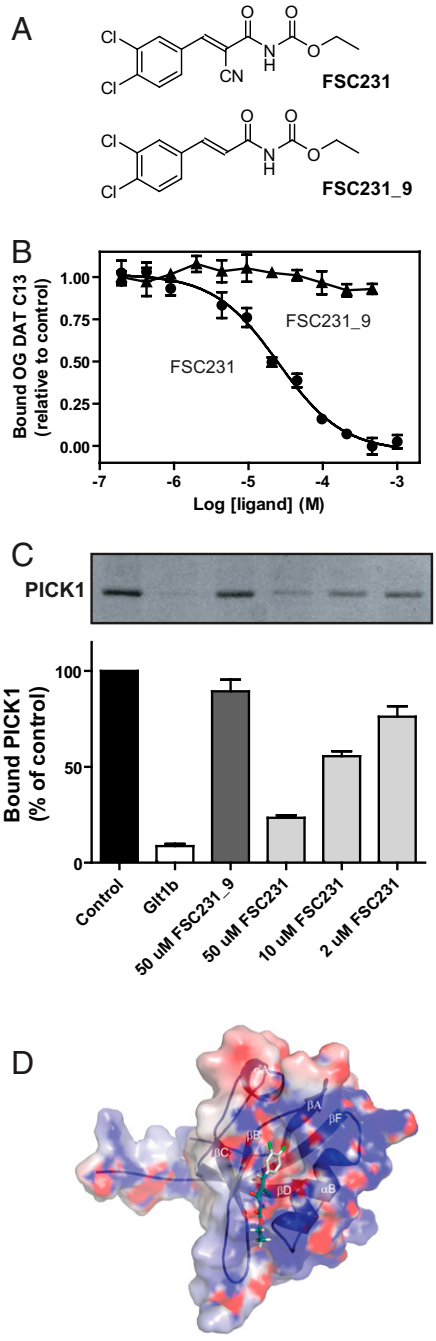

Fig. 1. FSC231 dose-dependently inhibits peptide binding to the PICK1 PDZ domain. (*A*) Chemical structure of FSC231 and the analog FSC231_9 lacking the cyano group. (*B*) FP competition curves for FSC231 and FSC231_9 using a fixed concentration of Oregon Green-labeled DAT peptide (OG-DAT C13, ~30 nM) and incubation (15 min) together with purified PICK1 and indicated compounds before FP measurements. Data are shown as bound relative to maximum bound OG-DAT C13 (means of triplicates ± SE) and representative of n = 9 (FSC231) and n = 3 (FSC231_9). (*C*) Pull-down of PICK1 by a C-terminal DAT GST fusion protein (GST DAT C24) is dose-dependently blocked by FSC231 but not by FSC231_9. (*Upper*) Representative BlueGel-stained SDS gel. (*Lower*) Quantified and pooled data from three independent experiments (means ± SE, n = 3). Sixty micromolars of GLT1b C10 peptide and 50 μM of FSC231_9 were used as positive and negative controls, respectively. (*D*) FSC231 binds in the ligand-binding groove of the PICK1 PDZ domain. The PDZ structure is colored by electrostatic potential calculated using Delphi software. Regions of negative potential are in red (at the −4 kT level); regions of positive potential are in blue and displayed at the +4 kT level. The FSC231 compound in mode 1 is rendered in a shaded representation.

term potentiation (LTP) (23–26). Importantly, FSC231, but not FSC231_9 lacking the cyano group, was able to inhibit binding of a fluorescently labeled C-terminal GluR2 peptide to the PICK1 PDZ domain with a potency similar to that seen for inhibition of DAT peptide binding [9.8 μM (9.1; 11 μM), $n = 3$] (Fig. S3*A*). Next we compared the potency of FSC231 directly to the potencies of the endogenous peptides. For GluR2, it is believed that PKCα-mediated phosphorylation of Ser880 (P_{-3} position) prevents the interaction of GluR2 with GRIP1, another PDZ interactions partner of GluR2, thereby shifting the GluR2 toward interaction with PICK1; hence, GluR2 phosphorylated at position 880 probably represents the physiologically relevant ligand (27). GluR2 wild type displayed a potency equal to that of FSC231 whereas the phosphorylated peptide (GluR2 p880) displayed two- to threefold lower potency (Fig. S3*B*).

To address whether FSC231 shows specificity for the PICK PDZ domain, we established FP binding assays for three canonical PDZ domains of PSD-95 (PDZ1–3) and for two PDZ domains of GRIP1 (PDZ4–5) (8). FSC231 in concentrations up to 1 mM was unable to compete for binding of a fluorescently tagged, C-terminal NMDA receptor (NR2b subunit) peptide to PDZ1 and -2 and for binding of a C-terminal cysteine-rich PDZ-binding protein (CRIPT) to PDZ3 (Fig. S4). Similarly, FSC231 could not displace a fluorescently tagged C-terminal GluR2 peptide from PDZ domains 4 and 5 of GRIP1 (Fig. S5).

FSC231 Inhibits the Interaction Between GluR2 and PICK1 in Hippocampal Neurons. To test whether FSC231 was able to pass the cell membrane and compete for intracellular PICK1 PDZ interactions in living cells, we measured fluorescence resonance energy transfer (FRET) in COS7 cells between PICK1, fused at the N terminus to enhanced yellow fluorescent protein (eYFP–PICK1), and the C-terminal 29 residues of GluR2, fused at the N terminus to enhanced cyan fluorescent protein (eCFP–GluR2 C29) (28). The FRET signal between eCFP–GluR2 C29 and eYFP–PICK1 was higher than that in the negative control, coexpressed eCFP and eYFP, and lower than that seen for the positive control, a fusion protein between eCFP and eYFP (eCeYFP) (Fig. 2*A*). Incubation with 50 μM FSC231, but not FSC231-9, significantly reduced the eCFP–GluR2/eYFP–PICK1 FRET signal (Fig. 2*A*), indicating that the compound was able to cross the cell membrane and block the interaction between the GluR2 C terminus and the PICK1 PDZ domain.

This was further confirmed in coimmunoprecipitation (co-IP) experiments performed to test whether FSC231 could inhibit the interaction between endogenously expressed PICK1 and GluR2. In extracts from hippocampal neurons, GluR2 was present in immunoprecipitates obtained with mouse anti-PICK1 antibody, and in agreement with an inhibition of the PICK1/GluR2 interaction, significantly less GluR2 was coimmunoprecipitated in extracts from neurons pretreated with 50 μM FSC231 (Fig. 2*B*). Pretreatment with FSC231-9 did not decrease GluR2 co-IP (normalized co-IP ~1.3, mean of $n = 2$). It is interesting to note that, in extracts from transfected HEK293 cells, FSC231 also inhibited co-IP of PICK1 with the metabotropic glutamate receptor mGluR7 (Fig. S6), another PICK1 interaction partner (29), further substantiating the ability of FSC231 to block the interaction between the PICK1 PDZ domain and its binding partners in live cells.

FSC231 Accelerates GluR2 Recycling After NMDA Receptor-Induced Internalization. PICK1 has been shown to promote intracellular accumulation of GluR2 in response to NMDA receptor activation either by stimulating GluR2 internalization or inhibiting its recycling (30, 31). We tested whether FSC231 was able to block the inhibitory effect of PICK1 on GluR2 recycling by expressing GluR2 tagged at the N terminus with the pH-sensitive green fluorescent protein variant, pHluorin (pH-GluR2) in hippocampal neurons (31). As shown before (31), pH-GluR2 recycled

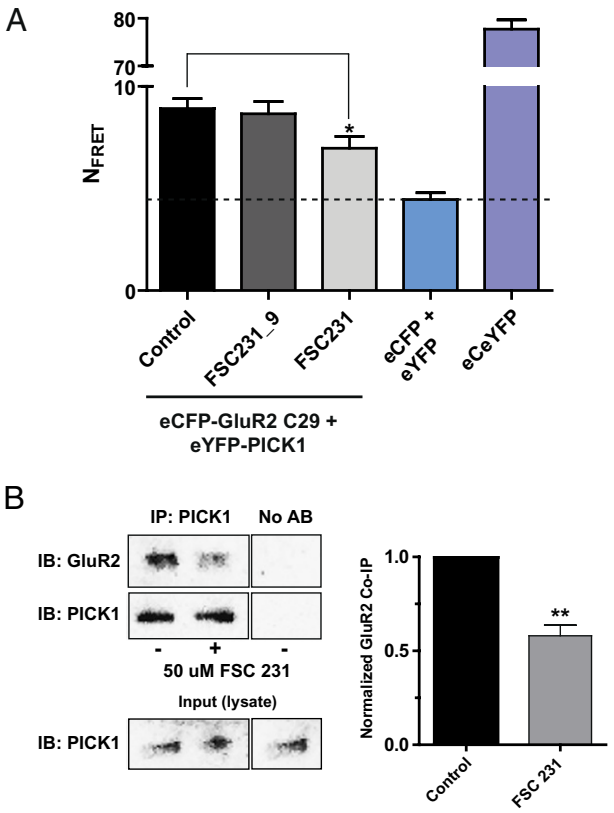

Fig. 2. FSC231 blocks binding between GluR2 and PICK1 in cells. (*A*) FSC231 inhibits FRET between eCFP–GluR2 C29 and eYFP–PICK1. Normalized FRET efficiency (N_{FRET}) is given for cotransfected eCFP–GluR2 C29 and eYFP–PICK1 without treatment (control) in response to 50 μM FSC231_9 or in response to 50 μM FSC231. As controls, we used eCeYFP (a covalent fusion of eCFP and eYFP) and cotransfection of eCFP and eYFP. Data are from three experimental days (means ± SE, $n = 3$). (*$P < 0.05$, ANOVA, post-hoc Bonferroni's test for multiple comparisons). (*B*) Co-IP of GluR2 with PICK1 in hippocampal neurons is inhibited by FSC231. PICK1 was immunopreciptated (IP) with rabbit anti-PICK1 antibody from extracts of hippocampal neurons pretreated with 50 μM FSC231 or vehicle. (*Top left*) Representative SDS–PAGE followed by immunoblotting (IB) with mouse anti-GluR2 antibody (IB: GluR2) shows diminished co-IP of GluR2 by FSC231. (*Right*) Normalized GluR2 co-IP after quantification by densitometry, mean ± SE, $n = 5$, **$P < 0.002$, one-sample *t* test). IB with mouse anti-PICK1 antibody (IB: PICK1) showed no change in PICK1 IP (*middle left*) or PICK1 input (*bottom left*) in response to FSC231.

back to the cell surface after NMDA receptor-induced internalization, and in agreement with inhibiting the function of PICK1, 50 μM FSC231 accelerated pH-GluR2 recycling without significantly affecting the amplitude of internalization (Fig. 3).

We also employed a two-color single-cell assay based on immunolabeling that we used to study trafficking of endogenously expressed GluR2 in response to direct activation of protein kinase C (PKC) and thus independently of NMDA receptor activation. Consonant with the described role of the GluR2/PICK1 interaction in PKC-mediated GluR2 redistribution (32), FSC231 significantly inhibited intracellular accumulation of GluR2 in response to phorbol 12-myristate 13-acetate (Fig. S7).

FSC231 Inhibits LTD and LTP in CA1 Hippocampal Neurons. Blocking the PICK1 PDZ domain with a C-terminal peptide of the GluR2 C terminus can inhibit hippocampal and cerebellar LTD expression (23, 24). To assess whether the inhibition of PICK1 by FSC231 also would translate into an effect on LTD, we examined its effects in CA1 hippocampal neurons from acute slices. Pairing a train of 900 stimulations at a frequency of 1 Hz with a depolarization of the

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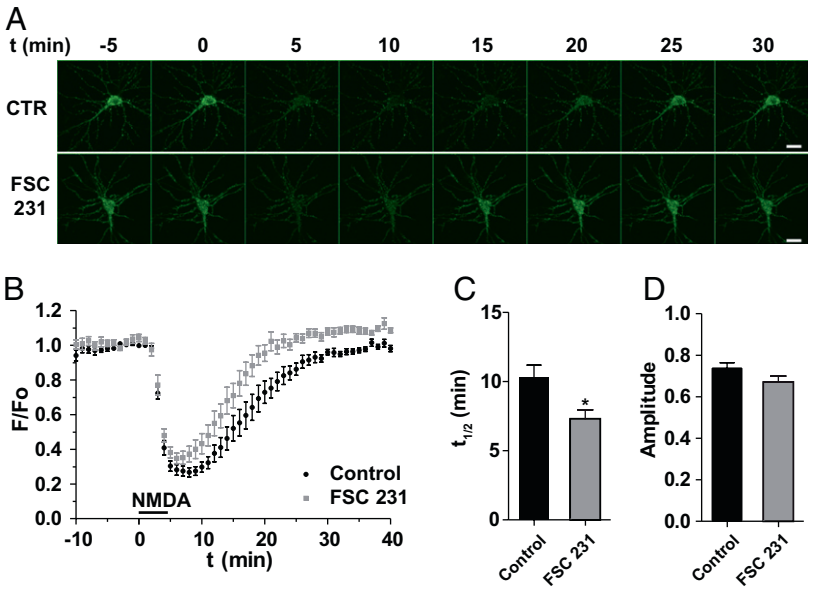

Fig. 3. FSC231 accelerates pHluorin-GluR2 recycling in CA1 hippocampal neurons. The pH-sensitive green fluorescent protein variant, pHluorin, was tagged to the N terminus of GluR2 (pH-GluR2) and transfected into hippocampal neurons. Internalization of pH-GluR2 was induced with 20 μM of NMDA for 5 min and fluorescence was recorded during the subsequent recovery period by confocal microscopy. (*A*) Representative series of images from control neurons and neurons treated with 50 μM of FSC231. (Scale bars: 10 μm.) (*B*) Representative time course of the average pHluorin-GluR2 fluorescence intensity (F) relative to initial fluorescence (F_0) from neurons treated with FSC231 and vehicle-treated control. (*C*) Average recycling half time ($t_{1/2}$ is the period from maximum endocytosis to 50% recycling) after NMDA washout and (*D*) average fluorescence intensity amplitude following NMDA stimulation (both means ± SE, n = 12 from four different transfections, $^*P < 0.02$, unpaired t test).

postsynaptic cell to −40 mV resulted as predicted (24) in a robust and long-lasting LTD (Fig. 4*A*). Inclusion of FSC231 (50 μM) in the intracellular solution of the patch pipette significantly reduced LTD (Fig. 4, *A*, *B*, and *D*). This reduction in LTD was similar to that seen for a C-terminal peptide of the GluR2 C terminus EVKI (NVYGIEEVKI) known to disrupt the PICK1–GluR2 interaction (Fig. 4, *B* and *C*). No reduction was seen in response to the control peptide SVKE (NVYGIESVKE), which does not bind PICK1 (Fig. 4, *B* and *C*).

Recent data have suggested a putative role of PICK1 in NMDA receptor-dependent LTP as well; e.g., LTP was absent in acute slices from PICK1 knock-out mice (26). Accordingly, we tested the effect of FSC231 (50 μM) on LTP expression in CA1 neurons in acute slices. Pairing a train of 50 stimulations at a frequency of 1 Hz with a depolarization of the postsynaptic cell to −5 mV resulted in a robust and long-lasting LTP (Fig. 4*E*). Inclusion of FSC231 (50 μM) in the intracellular solution significantly reduced LTP (Fig. 4, *E* and *F*). Finally, we tested the effect of FSC231 on the rectification index and the basal NMDA receptor/AMPA receptor ratio. FSC231 had no effect and thus did not affect the basal synaptic properties (Fig. S8). Correspondingly, the basal synaptic properties were previously shown to be unaltered in acute slices from PICK1 knock-out mice as compared to wild type (26) (*SI Note 3*).

Discussion

Insight into cellular protein networks has increased dramatically in recent years, rendering such protein–protein interactions increasingly interesting as putative targets for therapeutic intervention. This is particularly tempting for protein–protein interactions in the CNS where organization of larger multiprotein signaling complexes in distinct compartments is highly critical for neuronal function (7). Due to their key roles in the formation of these complexes, PDZ domains are predicted to be attractive targets because their well-defined binding crevices are likely to accommodate nonpeptide small-molecule ligands (6).

In line with this, we have identified a small-molecule inhibitor (FSC231) of the PICK1 PDZ domain. We focused on PICK1 because it is an important dimeric scaffolding protein widely expressed in the CNS (33) and because recent studies have suggested that the PICK1 PDZ domain might be an appealing drug target in relation to at least three cases involving dysfunction of AMPA receptor regulation and representing three unmet medical needs. In relation to neuropathic pain, it was observed that disruption of the interaction with the AMPA receptor by intrathecal injection of membrane-permeable PICK1-specific peptides alleviated neuropathic reflex sensitization induced by chronic constriction injury (13). For excitotoxicity following cerebral trauma, PICK1-binding peptides preserved surface GluR2 expression, which in turn attenuated AMPA-receptor-mediated neuronal toxicity (14). For cocaine sensitization, it was observed that i.p. injection of a membrane-permeable PICK1-specific peptide before a single dose of cocaine abolished development of synaptic plasticity in the ventral tegmental area (15). This plasticity is thought to underlie cocaine-induced behavioral sensitization and conditioned place preference, both considered core components of addiction (34).

Molecular modeling and computational simulations indicated how FSC231 can be accommodated within the PDZ-binding crevice in a binding mode that resembles that suggested for a small-molecule inhibitor of the AF6 PDZ domain (11). The affinity of FSC231 for the crevice was ~10 μM, which is essentially identical to that seen for the C-terminal peptide of the GluR2 subunit of the AMPA receptor and a few-fold higher than the affinity of the corresponding phosphorylated peptide (pSer880), believed to be the physiological ligand (27). According to both FRET and coimmunoprecipitation experiments, FSC231 was able to cross the plasma membrane and inhibit the interaction between GluR2 and PICK1 in living cells. Moreover, FSC231 interfered with GluR2 trafficking, which is consistent with inhibiting the role of PICK1 in slowing down GluR2 recycling and/or accelerating GluR2 internalization. Finally, FSC231 was shown to inhibit both LTD and LTP expression in CA1 hippocampal neurons, which is consistent with inhibition of PICK1's bidirectional effect of PICK1 on synaptic plasticity (26).

Only a few small-molecule PDZ domain inhibitors have been described in the literature (10, 11, 16, 17, 35, 36). Most of these have been designed on the basis of selected scaffolds rather than high-throughput screening of compound libraries including inhibitors of, e.g., dishevelled [affinity up to ~10 μM (10, 36)] and NHERF1 (affinity ~15 μM) (35). Although a putative inhibitor of the nNOS/PSD-95 interaction was recently identified by screening ~150,000 compounds (37), high-throughput screening has in general appeared very challenging; for example, screening of more than 100,000 compounds against Mint1 did not lead to identification of any specific binders (18). Nonetheless, the data from the present as well as previous studies indicate that indeed small-

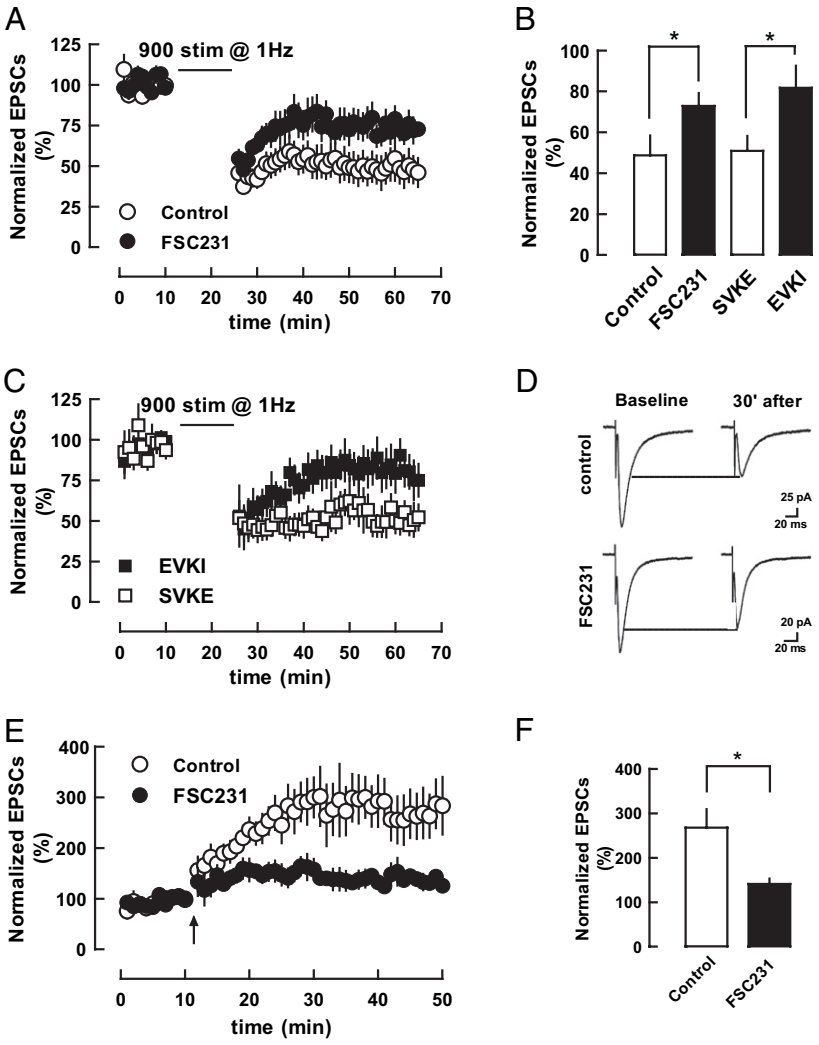

Fig. 4. FSC231 attenuates LTD and LTP in CA1 hippocampal neurons. (*A*) LTD expression in CA1 hippocampal neurons was significantly reduced when FSC231 was included in the patch pipette (solid circles) as compared to saline control (open circles) tested in parallel. LTD was induced by pairing a train of 900 stimulations at a frequency of 1 Hz with a depolarization of the postsynaptic cell to −40 mV. (*B*) Average normalized excitatory postsynaptic currents (EPSCs) of the experiments presented in *A* and *C* (means ± SE of n = 7–11, $*P < 0.05$ unpaired t test). (*C*) The PICK-specific peptide (EVKI, solid circles) significantly reduced LTD compared to a nonbinding peptide (SVKE, open circles) tested in parallel. (*D*) Representative EPSCs with or without treatment with FSC231 before and 30 min after application of LTD-inducing stimuli. (*E*) LTP in CA1 hippocampal neurons was significantly reduced when FSC231 was included in the patch pipette (solid circles) as compared to saline control (open circles). LTP was induced by pairing a train of 50 stimulations at a frequency of 1Hz with a depolarization of the postsynaptic cell to −5 mV. (*F*) Average normalized EPSCs of the experiment presented in *D* (means ± SE of n = 4–5, $*P < 0.05$ unpaired t test).

molecule inhibitors of PDZ domains can be identified and, similar to our FSC231 compound, are likely to display pharmacological activity that makes them putative leads in drug discovery efforts. An important remaining task is to improve further the affinity, preferentially into the nanomolar range. Given that screening of phage libraries has identified short peptides with affinities in this range, e.g., for the Erbin PDZ domain (38), it is not unlikely that the nonpeptide small-molecule ligands can achieve such high affinity. Moreover, it is of interest that peptidomimetics have been developed against the PDZ domain of α1-syntrophin and PDZ1 and PDZ2 of PSD-95 with affinities of ≈0.5–1 μM (39, 40).

PDZ domain selectivity also may be of some concern in the design of inhibitors, given the structural conservation and the fact that the human genome encodes up to 540 different PDZ domains. Our results for FSC231 suggest, however, that selectivity is achievable (at least among the domains tested), as we found that FSC231 did not affect binding of peptide ligands to the PDZ domains of PSD-95 and GRIP, demonstrating that it is not a general PDZ inhibitor. In addition, our computational studies suggested a highly unfavorable binding mode for FSC231 upon docking into yet other PDZ domains, such as that of AF6 and Dishevelled.

In summary, we have demonstrated that screening of a compound library can successfully identify small-molecule nonpeptide compounds that fit in the PDZ-binding crevice. The compound identified (FSC231) represents a previously undescribed small-molecule inhibitor of the PICK1 PDZ domain and displays an affinity similar to, or even higher than, that observed for the endogenous C-terminal peptide ligands. Importantly, we show that FSC231 exhibits both selectivity for the PICK1 PDZ domain and interesting pharmacological activity. It should be borne in mind that blocking the PICK1 PDZ domain might not always be advantageous; i.e., inhibiting the interaction between PICK1 and mGluR7 can potentially cause absence-like seizures (41). Nevertheless, it will still be highly interesting in future experimental efforts to further evaluate FSC231 as a putative lead compound for the development of new therapeutics against, e.g., neuropathic pain and cocaine addiction.

Experimental Procedures

Molecular Biology and Protein Purification. The molecular biology procedures and constructs are described in *SI Methods*. GST–PICK1 and N-terminally polyhistidine tagged PSD-95 PDZ domains were expressed in *Escherichia coli* BL21(DE3) pLysS (Novagen) and purified as described (19, 39, 40) (*SI Methods*).

FP Competition and Screening Assays. The PICK1 competition and screening FP assays were performed according to described methods (19) (*SI Methods*).

GST Pull-Down Assay and co-IP Experiments. GST pull-down assays were carried using a fusion between GST and the 24 C-terminal residues of the dopamine transporter (GST DAT C24) (20). Co-IPs were performed on cultured hippocampal neurons or transfected HEK293 cells as described in *SI Methods*.

Molecular Modeling, Molecular Dynamics Simulations, and Free Binding Energy Calculations. These procedures are described in *SI Methods*.

FRET. FRET experiments were performed using the "three-filter method" as described (28) (*SI Methods*).

Phluorine-GluR2 Recycling Assay. The assay was performed as described (31) (*SI Methods*).

Electrophysiology. Experiments on CA1 pyramidal cells in hippocampal slices were performed as described in *SI Methods*.

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