PICK1 INTERACTS WITH α7 NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS AND CONTROLS THEIR CLUSTERING

Abbreviated title: PICK1 controls α7 nAChR clustering

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Abstract

Central to synaptic function are protein scaffolds associated with neurotransmitter receptors in clusters. \( \alpha_7 \) neuronal nicotinic acetylcholine receptors (nAChRs) modulate network activity, neuronal survival and cognitive processes in the CNS, but protein scaffolds that interact with these receptors are unknown. Here we show that the PDZ-domain containing protein PICK1 binds to \( \alpha_7 \) nAChRs and plays a role in their clustering. PICK1 interacted with the \( \alpha_7 \) cytoplasmic loop in yeast in a PDZ-dependent way, and the interaction was confirmed in recombinant pull-down experiments and by co-precipitation of native proteins. \( \alpha_7 \) and PICK1 co-localized in clusters at the surface of GABAergic interneurons in dissociated hippocampal cultures. Expression of PICK1 caused decreased \( \alpha_7 \) clustering on the surface of the interneurons in a PDZ-dependent way. These data show that PICK1 negatively regulates surface clustering of \( \alpha_7 \) nAChRs on hippocampal interneurons, which may be important in inhibitory functions of \( \alpha_7 \) in the hippocampus.
Introduction

Molecular scaffolds organize synaptic structures and downstream signaling processes. Among nAChRs, members of the PSD95 family interact with α3 and β4 subunits in the peripheral nervous system (Conroy et al., 2003; Parker et al., 2004), but no intracellular proteins regulating clustering of nAChRs have been identified in the central nervous system (CNS), yet. α7 nAChRs are prominent nAChRs and constitute α-bungarotoxin-(α-BT)-binding sites widely expressed throughout the CNS (Jones et al., 1999). They are important in learning, attention, nicotine addiction, and involved in neurodegenerative diseases and schizophrenia (Jones et al., 1999; Martin et al., 2004; O'Neill et al., 2002). α7 nAChRs are highly permeable for calcium (Seguela et al., 1993), present at synaptic and extrasynaptic sites (Fabian-Fine et al., 2001; Kawai et al., 2002; Levy and Aoki, 2002; Shoop et al., 1999) and have numerous functions in cell survival and synaptic plasticity (Dajas-Bailador and Wonnacott, 2004), implying specific interaction with appropriate signaling and scaffolding molecules (Berg and Conroy, 2002; Huh and Fuhrer, 2002). Src-family kinases (SFKs) have recently been found to associate with α7 nAChRs, causing α7 phosphorylation and decreased receptor activity (Charpantier et al., 2005). Unlike in the case of the neuromuscular AChR, however (Sadasivam et al., 2005; Willmann et al., 2006), SFKs do not seem to control clustering of α7 nAChRs (Wiesner and Fuhrer, 2006).

In the hippocampus, which receives rich cholinergic innervation from the septal complex, α7 nAChRs are highly expressed in GABAergic interneurons where they form postsynaptic clusters (Kawai et al., 2002), mediate cholinergic synaptic input (Alkondon et al., 1998; Frazier et al., 1998) and regulate inhibition within the hippocampal network (Alkondon et al., 1997; Jones and Yakel, 1997). Activation of these α7 receptors blocks concurrent STP and LTP induction in pyramidal cells (Ji et al., 2001). Inhibition of pyramidal neurons by postsynaptic α7 nAChRs on interneurons also underlies hippocampal auditory gating, linking α7 to the pathogenesis of schizophrenia (Martin et al., 2004; Ripoll et al., 2004). Neuregulin, neurotrophins and NMDA receptor activity increase interneural α7 levels or clustering in hippocampus (Kawai et al., 2002; Liu et al., 2001) whereas raft-like lipid microdomains are important in α7 clustering in neurons of the ciliary ganglion (Bruses et al., 2001) - but in all these cases the intracellular proteins mediating or modulating α7 clustering remain unknown.

Here we identify PICK1 as a first scaffolding protein that interacts with α7 nAChRs. PICK1 was originally isolated as a binding protein of protein kinase C (PKCα) (Staudinger et al., 1995),
and PICK1 is important in synaptic targeting and clustering of other neurotransmitter receptors. Presynaptically, PICK1 binds to the C-terminus of mGluR7a and causes receptor clustering and phosphorylation by PKC (Boudin et al., 2000; Dev et al., 2000). Postsynaptically, PICK1 binds to and clusters AMPA (Xia et al., 1999) and kainate receptors through its PDZ domain (Hirbec et al., 2003). Furthermore, PICK1 influences glutamate receptor transport processes suggesting a role in the release of receptors from synaptic anchors and in receptor transport from the synaptic membrane towards endocytotic pathways (Perez et al., 2001; Steinberg et al., 2006; Terashima et al., 2004).

We find that the α7-PICK1 interaction involves the PDZ domain of PICK1 and a segment of the α7 intracellular loop. Interaction is shown in the yeast two-hybrid system and is confirmed in precipitation assays using recombinant and native proteins and by protein colocalization in hippocampal GABAergic interneurons. Interestingly, PICK1 negatively regulates clustering of α7 receptors in these interneurons, suggesting that PICK1 may play a specific role in α7-mediated inhibition of the hippocampal network.

**Results**

**Identification of PICK1 as an α7 interaction partner using the yeast two-hybrid system**

To search for intracellular molecules that interact with α7 nAChRs, we used the cytoplasmic loop of α7 as bait to screen a rat brain cDNA library using the yeast two-hybrid (YTH) technique (Fields & Song, 1989) (bait 1, aa 332-467, Fig. 1A). This loop is situated between transmembrane domains 3 and 4 and comprises most of the cytoplasmic portion of the α7 receptor. Positive candidates were verified by cotransformation of bait and prey clones into yeast and repeated lift filter assays. Among others, we identified two clones that encode full-length PICK1 (aa 1-417, Fig. 1A), showing that the α7 loop interacts with PICK1 in yeast.

Besides the homopentameric α7 nAChRs that form α-BT binding sites, heteropentameric α4/β2 nAChRs are abundant in brain (Lindstrom et al., 1995). We characterized the specificity of the α7 nAChR-PICK1 interaction by examining the binding of PICK1 to the cytoplasmic loop sequence of the α4 and β2 nAChR in the YTH system. PICK1 did not interact with α4 or β2 nAChR subunits, illustrating the specificity of the PICK1-α7 loop interaction in yeast (Fig. 1A).

A C-terminal segment of the α7 loop and the PDZ-domain of PICK1 mediate binding
To map the site of interaction between α7 nAChR and PICK1, various bait constructs of the α7 cytoplasmic loop were tested for interaction with full-length PICK1 (Fig. 1A). Deleting the C-terminal region of the α7 loop bait eliminated the interaction (baits 7, 8), while baits containing this region still interacted with PICK1 (baits 9, 10). These data show that a C-terminal segment (aa 429-467; bait 9) close to the TM4 domain of α7 nAChR is necessary and sufficient to bind to PICK1 in yeast.

PICK1 comprises three major structural domains important for protein interactions, an N-terminal PDZ domain (aa 20-110), a coiled-coil domain (aa 139-166) and a C-terminal acidic region (aa 380-390) (Staudinger et al., 1997; Xia et al., 1999) (Fig. 1B). Previous studies indicated that these domains are important for clustering and synaptic localization of PICK1 (Boudin and Craig, 2001), the PDZ domain being necessary for interactions with various neurotransmitter receptors (Boudin et al., 2000; Hirbec et al., 2003; Xia et al., 1999). To determine whether the PICK1 PDZ domain also mediates binding to α7 nAChRs, we used two additional PICK1 prey constructs, one containing the PDZ domain only (aa 1-126), the other lacking it (aa 126-417, Fig. 1B). None of the α7 nAChR baits interacted with PICK1 lacking its PDZ domain (Fig. 1A). Baits 1, 9 and 10, which interacted with the full-length PICK1, also interacted with the short prey containing PICK1’s PDZ domain only (Fig. 1A). This shows that the PDZ domain of PICK1 is both necessary and sufficient for interaction with the α7 nAChR loop.

In general, protein interactions mediated by PDZ-domains are of great versatility, as PDZ domains bind to small C-terminal peptides (through class I, II and III binding motifs), internal protein segments, other PDZ domains or even lipids (Nourry et al., 2003). We analyzed the sequence of the α7 cytoplasmic loop for potential class I, II, and III PDZ-binding motifs and identified eight putative motifs in bait 1, two of which are also present in the shortest PICK1-interacting bait (bait 9, Fig. 1C). We point-mutated these two putative PDZ-binding motifs singly or together in bait 1 and 9 (Fig. 1C). Mutation was done at the 2\textsuperscript{nd} and 4\textsuperscript{th} amino acids of the consensus by replacement with alanine, to inactivate the motif (Nourry et al., 2003). All mutated baits still bound normally to full-length PICK1 through its PDZ domain (Fig. 1C). Thus the α7 nAChR-PICK1 interaction reported here does not depend on α7 sequences similar to class I, II and III PDZ-binding motifs. The interaction does depend on PICK1’s PDZ domain, however, and on an internal segment of the α7 receptor contained within its cytoplasmic loop close to TM4.

Interaction of recombinant α7 and PICK1 in heterologous cells

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The interaction of α7 nAChR and PICK1 was further examined by recombinant protein pull-down experiments and immunoblotting. COS cells or bacteria were transfected with either full-length α7 nAChR or tagged (myc, His) PICK1 expression vectors. Cell lysates were incubated with glutathione-S-transferase (GST) fusion proteins immobilized to glutathione-Sepharose beads (Fig. 2). These fusion proteins contained either full-length PICK1 (GST-PICK1) or the cytoplasmic loop of α7 nAChR (GST-α7loop), or the cytoplasmic loop of α4 nAChR (GST-α4loop) as a control. These assays showed that GST-PICK1 beads precipitated α7 nAChRs from COS cells (Fig. 2A), while GST-α7loop beads pulled down myc-PICK1 from COS lysates (Fig. 2B) and also His-PICK1 from bacteria (Fig. 2D). In contrast, GST-α4loop beads did not pull down myc-PICK1 from COS cells indicating the specificity of the interaction between recombinant α7 nAChR and PICK1. These results confirm the YTH data and demonstrate the interaction of the α7 loop and PICK1.

Association of native PICK1 with α7 nAChRs in brain

We next performed co-precipitation experiments to test for interaction between the native proteins in rat brain. From synaptosome preparations of adult rat hippocampus, α7 nAChRs were first precipitated with α-BT coupled to sepharose beads according to established protocols (Drisdel and Green, 2000; Fuhrer and Hall, 1996). Samples were analyzed by PICK1 immunoblotting, revealing the presence of PICK1 in the α7 precipitates (Fig. 3A). The presence of α7 nAChR after α-BT-precipitation was verified using anti-α7 antibodies (Fig. 3A). Pre-incubation with free excess toxin abolished the α7 signal and strongly decreased levels of PICK1 signal, demonstrating specific α7-PICK1 association of native proteins in brain (Fig. 3A). In addition, the specificity of the α-BT-precipitation and the presence of PICK1 in the α7 precipitates were demonstrated by nicotine-competition, which eliminated the α7 nAChR signal and strongly reduced the PICK1 signal in the corresponding Western blots (Fig. 3B).

We also precipitated α7 nAChRs from synaptosomes using anti-α7 antibodies and again detected associated PICK1 by immunoblotting (Fig. 3C, left). Omitting antibodies or synaptosomes from the precipitation eliminated the PICK1 signal (Fig. 3C, left). α7-precipitation from synaptosomal preparations of cerebellum or cerebral cortex showed reduced signals compared to hippocampus (Fig. 3C, left) as expected from the high relative abundance of α7 in hippocampus (Seguela et al., 1993). To further assess the specificity of the α7
immunoprecipitation we used non-immune IgG as a control and found no associated PICK1 signal (Fig. 3C, right).

To further illustrate specificity of α7-PICK1 interaction, we probed α7 immunoprecipitates from hippocampal synaptosomes for the presence of other synaptic proteins, GluR2 (an AMPA receptor subunit) and members of the PSD95 family (using pan-PSD95 antibodies). Neither GluR2 nor PSD95-family members were associated with α7, but were clearly visible in the starting synaptosomal preparation (Fig. 3D). Taken together, the co-precipitation experiments demonstrate that native α7 nAChRs are specifically associated with PICK1 in the hippocampus.

**PICK1 partially colocalizes with α7 in heterologous cells but does not induce α7 clustering**

PICK1 has been shown to cluster AMPARs (Dev et al., 1999; Xia et al., 1999) and mGluR7a (Boudin and Craig, 2001; Boudin et al., 2000) in heterologous expression systems. To examine if PICK1 could induce α7 nAChR clustering, we transfected PICK1 and α7 into COS cells, HEK 293T cells and the human neuroblastoma SH-SY5Y cell line, and analyzed α7 and PICK1 distribution by immunofluorescence staining (Fig. 4). In COS cells, we observed largely diffuse intracellular α7 and PICK1 immunofluorescence with a concentration in the perinuclear area, and occasionally some PICK1 clusters (Fig. 4A, and data not shown). The same result was seen whether PICK1 and α7 were expressed together (Fig. 4A, lower section) or singly (Fig. 4A, upper section). The perinuclear diffuse signal showed partial overlap of α7 with PICK1 (Fig. 4A, panel f, yellow in overlay). Likewise, in HEK 293T cells, α7 immunofluorescence was diffusely distributed and did not reveal clusters (Fig. 4B) whether or not PICK1 was co-expressed (Fig. 4B and data not shown). PICK1 formed more clusters than in COS cells (Fig. 4B, panel b), also when α7 was not co-expressed (data not shown), and the PICK1 clusters did not overlap with clusters of α7.

In SH-SY5Y cells stably expressing α7, the α7 nAChRs form functional channels and bind α-BT (Charpantier et al., 2005; Cooper and Millar, 1997; Peng et al., 1994). We transfected these cells with a PICK-EYFP fusion construct. Rhodamine-α-BT staining of intact cells revealed surface clusters of α7 (Fig. 4C). The PICK1-EYFP signal appeared diffuse and as clusters, and some of these clusters overlapped with the α7 clusters (Fig. 4C, yellow in overlay). α7 clustering was identical in cells not transfected with PICK1-EYFP (data not shown). To confirm the specificity of the α7 nAChR signal on SH-SY5Y cells, rhodamine-α-BT incubation was performed in the presence or absence of nicotine. Nicotine-competition drastically reduced binding of rhodamine-α-BT, demonstrating the specificity of the α-BT signal for α7 clusters (Fig. 4D).
Taken together, these data indicate that PICK1 does not induce clustering of α7 nAChRs, although PICK1 itself, in agreement with previous studies (Xia et al., 1999), can form clusters in heterologous cells. α7 and PICK1 partially overlap diffusely in COS and HEK 293T cells and in clusters in SH-SY5Y cells, consistent with their interaction.

**PICK1 colocalizes with α7 nAChR in clusters in rat hippocampal GABAergic interneurons**

We next assessed the subcellular distribution of α7 receptors and PICK1 in neurons using immunofluorescence microscopy. Primary cultures of rat hippocampal neurons were stained during the second and third week *in vitro* (Fig. 5). Fluorescent α-BT, added to intact cells, specifically labeled surface α7 clusters along membranes (Fig. 5), confirming the pattern demonstrated previously (Kawai et al., 2002). The labeling was specific as it was blocked by adding excess unlabeled α-BT or nicotine (not shown; but see Kawai et al., 2002). α7 clusters were easily detectable on processes proximal and distal to the soma, and often appeared grouped into larger aggregates on the cell soma (Fig. 5).

α-BT-labelled clusters occurred on only a subset of neurons. To determine their identity, we double-labeled cells with α-BT and antibodies recognizing GAD (glutamic acid decarboxylase) or VGAT (vesicular GABA transporter) (Fig. 5). 5-10% of all neurons were GAD- or VGAT-positive, revealing thus a low density of GABAergic interneurons. Consistent with a previous report (Kawai et al., 2002), α7 nAChR clusters were only present on GAD- or VGAT-positive neurons and labeled most of these cells (Fig. 5). Furthermore, α7 nAChR clusters showed some overlap with VGAT- or GAD-immunoreactivity (IR) (Fig. 5) and also with GABA_A receptor α1 subunit-IR (data not shown), indicating that some of these α7 clusters are located at GABAergic synapses. Average density of α-BT-clusters in dendrites (Fig. 5, 6) was 15.0 clusters per 100 μm segment (averaged from 145 dendrite segments of 100 μm length from 40 cells of three independent cultures). This value is similar to published GABA_A receptor α2 subunit clusters apposed to GAD boutons (14.7 per 100 μm segment) (Brunig et al., 2002).

The synaptic localization of many interneuronal α7 clusters remains unclear in cultured hippocampal cells (Kawai et al., 2002) although some overlap with synaptotagmin label has been reported (Zarei et al., 1999). Extra- and perisynaptic α7 receptors are found in hippocampal and ciliary ganglion tissue sections (Fabian-Fine et al., 2001; Shoop et al., 1999). We performed pair-wise double-labels with α-BT and antibodies against bassoon, gephyrin and PSD-95 in our hippocampal cultures. The overlap of α-BT signal with these markers was very low (K. Baer and
Since cultured hippocampal cells lack cholinergic neurons, it is thus possible that many of our α7 clusters represent extrasynaptic receptors that, in vivo, may be recruited postsynaptically by cholinergic nerve terminals.

Double-labeling using α-BT and PICK1 antibodies showed a punctate distribution for both proteins in interneurons and indicated significant, although not exclusive, overlap of α7 nAChR and PICK1: 57.5 ± 17.1% of α-BT-clusters (mean±SD; in n=145 dendrite segments of 100 μm length from 40 cells of three independent cultures) colocalized with PICK1 clusters (Fig. 6). The PICK1 staining pattern is in good agreement with previous studies showing that PICK1 is enriched at synapses both pre- and postsynaptically, also in pyramidal cells (Torres et al., 2001; Xia et al., 1999). Collectively, our data demonstrate that α7 clusters are found on GABAergic hippocampal interneurons and that almost 60% of these clusters colocalize with PICK1.

**PICK1 reduces α7 nAChR surface clusters in interneurons**

To determine whether PICK1 controls clustering of α7 nAChRs at the surface of hippocampal interneurons, we expressed wild-type and PDZ-mutant PICK1 in neuronal cultures using Sindbis virus. To this end, bicistronic constructs used previously were employed (Terashima et al., 2004) to express PICK1 together with free EGFP, enabling the identification of infected neurons by EGFP fluorescence. Either wildtype PICK1 (WT) or mutant PICK1 (AA) were virally expressed. PICK1-AA contains two point mutations in the PICK1 PDZ domain that eliminate PDZ-dependent interactions (Terashima et al., 2004; Xia et al., 1999). As a control, Sindbis virus expressing only EGFP was used.

To analyze the effects of PICK1 on α7 nAChR surface clusters, we examined α7 cluster distribution using rhodamine-α-BT labeling following virus infection in 14 d-old hippocampal neurons. PICK1 expression, represented by EGFP fluorescence, was visible in the somata and processes of infected interneurons. A reduction in α-BT labeling of α7 nAChR at the surface was evident selectively in cells infected with PICK1-WT-EGFP virus (Fig. 7). Overexpressing EGFP only or the mutant PICK1-AA protein did not change the pattern of α7 surface clusters, showing that the functional PDZ domain of PICK1 is needed for an effect on α7 nAChR clusters.

The effects of PICK1 viral expression were quantitatively assessed comparing α-BT cluster levels within groups and per cellular region. On the soma of interneurons, clusters often appeared grouped into larger aggregates, as noted for Figure 5. Our data revealed a significant reduction in α7 α-BT clusters, measured as the cumulative α-BT signal per surface area, on the somata and
proximal dendrites of interneurons expressing WT PICK1 (Fig. 7). No effect on α7 clusters was seen in non-infected, EGFP- or PICK1-AA-EGFP-infected interneurons.

The effect of PICK1 on α7 nAChR clustering was confirmed by magnetofection to express PICK1 in interneurons. We transfected PICK1-EYFP (fusion protein) or EYFP constructs into hippocampal primary neurons (11 days in vitro) and examined α7 cluster distribution using rhodamine-α-BT. The results show, as in the case of virus-infected cells, that interneurons expressing PICK1-EYFP or EYFP have a healthy morphology indicating that PICK1 expression per se did not harm these cells. PICK1-EYFP was observed diffusely and in clusters, as shown previously for myc-tagged PICK1 in hippocampal cultures (Boudin and Craig, 2001). The amount of α7 surface clusters on dendrites of transfected interneurons again was measured as the cumulative α-BT signal per surface area. These data showed a significant reduction of the α-BT signal in interneurons expressing PICK1-EYFP compared to the control group of interneurons expressing EYFP (Fig. 8), thus confirming the results from viral expression (Fig. 7).

To further ascertain that PICK1 expression does not harm the cells causing non-specific redistribution of other surface receptors, we expressed PICK1-EYFP or EYFP constructs in cultured neurons using magnetofection and stained the neurons against GABA_A receptor α1 subunit together with VGAT as markers for interneurons (Fig. 9). The results show that the GABA_A receptor α1 subunit signal is not downregulated in interneurons after PICK1-EYFP expression compared to control EYFP expression. Taken together, these data demonstrate that overexpression of PICK1 in hippocampal GABAergic interneurons does not have a general effect on surface receptors, but specifically reduces surface clusters of α7 nAChRs.

Discussion

This study identifies the first synaptic scaffold protein, PICK1, that interacts with nAChRs in the CNS, exemplified by α7 nAChRs. We show that PICK1 binds to α7 in yeast, heterologous mammalian cells and hippocampal tissue. PICK1 colocalizes with α7 in clusters at the surface of hippocampal GABAergic interneurons and negatively regulates α7 nAChR clustering in these cells.

**PICK1 interacts with α7 nAChRs through its PDZ domain and an internal segment of the α7 loop**
Very little is known about protein interactions of α7 nAChRs. SFKs bind to the cytoplasmic loop of α7, phosphorylate the receptor and decrease its activity (Charpantier et al., 2005). Ric-3 has been identified as an effector of functional expression and maturation of various nAChRs, including α7 receptors, in vertebrates and invertebrates. Ric-3 protein associates with α7 subunits in a complex, although it remains unknown whether it directly binds to the α7 nAChR and where such a binding region would map within the α7 protein (Ben-Ami et al., 2005; Halevi et al., 2002; Lansdell et al., 2005; Williams et al., 2005).

We identify PICK1 as a binding partner for the α7 cytoplasmic loop, and our experiments strongly suggest that this represents a direct and specific interaction of the two proteins. Thus, we observe PICK1-α7 interaction in yeast, using the cytoplasmic loop of α7 as a bait. In recombinant pulldown experiments α7 loop fusion protein (GST) interacts with PICK1 protein that is either expressed in COS cells or in bacteria, and the interaction is also seen in the reverse case, using GST-PICK1 to pull down α7. Furthermore, interaction between PICK1 and α7 receptors is observed in the case of native proteins, because α-BT- or α7-antibody-precipitations bring down, in a specific fashion, PICK1 in lysates from brain and dissected hippocampus. In controls, the loops of other nAChR subunits do not interact with PICK, and α7 nAChRs do not associate with PSD95-proteins or GluR2 receptors. Finally, α7 nAChRs and PICK1 partially co-localize in heterologous cells and in clusters in GABAergic interneurons. The combination of these data strongly implies a direct and specific interaction between PICK1 and the α7 loop. An intermediate protein would have to exist in yeast, bacteria, COS cells and neurons; it would have to survive the GST protein purification on glutathione-sepharose, and this is very unlikely.

We mapped the involved binding regions in both α7 and PICK1. Whereas in α7, a C-terminal peptide of the intracellular loop was necessary and sufficient, binding in PICK1 was mediated by its PDZ domain. Although the α7 loop contains motifs similar to consensus binding motifs for PDZ-domains, these α7 sequences were not necessary to bind to the PDZ domain of PICK1. Thus the PDZ domain of PICK1 binds to an internal region in the α7 loop independent of consensus motifs.

PDZ domains of synaptic scaffolding proteins often bind to short motifs (Type I, II or III) at the intracellular C-terminus of transmembrane receptors (Nourry et al., 2003). In this manner, PICK1 interacts with AMPA receptor subunits, mGluR7a, kainite receptors and others, through Type I or Type II PDZ-binding motifs (Boudin and Craig, 2001; Boudin et al., 2000; Hirbec et al., 2003; Madsen et al., 2005; Torres et al., 2001; Torres et al., 1998; Xia et al., 1999). We expand
this range by introducing an interaction of PICK1’s PDZ domain with an internal protein segment in the cytoplasmic loop of α7. Although novel for PICK1, other PDZ domains are well known to bind to internal protein portions (Nourry et al., 2003). Internal recognition can be analogous to C-terminal interactions, i.e. according to the Type I, II or III consensus features (Gee et al., 1998), suggesting that many PDZ domains might recognize internal motifs if these are provided in the correct structural context (Harris and Lim, 2001). Nonetheless, internal peptides lacking any consensus features can also be ligands for PDZ domains. One example is the interaction of dishevelled with the receptor Frizzled (Wong et al., 2003), and the PICK1-α7 interaction reported here expands this category.

**PICK1 reduces clustering of α7 nAChRs at the surface of hippocampal GABAergic interneurons**

To address the role of PICK1 in clustering of surface receptors, two standard tools are most often applied: (i) expression of receptor and PICK1 in heterologous cells to assess whether PICK1 can induce receptor clustering and (ii) overexpression of PICK1 in neurons that endogenously express the receptor to determine whether PICK1 affects native receptor clusters (Torres et al., 1998; Xia et al., 1999) (Boudin and Craig, 2001; Boudin et al., 2000; Torres et al., 2001). These studies showed that in heterologous cells, PICK1 induces clustering of GluR2-containing AMPA-Rs, mGluR7a and others. The situation in neurons is more complex, as PICK1 can increase or decrease synaptic clustering of neurotransmitter receptors, depending on receptor subunits and neural cell type (Perez et al., 2001; Terashima et al., 2004; Torres et al., 2001). In our case, unlike any of the receptors described previously, PICK1 expression did not induce or affect clusters of α7 nAChRs in heterologous cells including SH-SY5Y, even though PICK1 itself was clustered, particularly in HEK 293T cells and SH-SY5Y cells. Yet, expression of PICK1 reduced clustering of α7 at the surface of GABAergic hippocampal interneurons. This reduction was a specific and most likely direct process because i), the reduction was seen by using two entirely different techniques to express PICK1, viral expression or magnetofection; ii) the reduction, in the same way as binding to α7 did, required an intact PICK1 PDZ domain, since the AA mutation or expression of GFP alone had no effect; iii) the reduction did not involve intercellular interactions, because only PICK1-transfected interneurons were affected rather than adjacent non-transfected interneurons; and iv) PICK1 expression did not affect surface clustering of GABA_A receptors in hippocampal interneurons demonstrating that PICK1 does not have a general effect on surface receptors, but rather specifically reduces α7 surface clusters.
Our experiments point toward a specific PICK1-α7 mechanism, mediated by binding between these proteins, that controls α7 clustering at the surface of hippocampal interneurons. PICK1 does not induce α7 nAChR clustering, but interacts with the receptor in clusters and negatively regulates or limits α7 clustering. This mechanism may depend on one or several proteins expressed in interneurons that bind(s) to the α7-PICK1 complex and effects its targeting. In such a manner, α7-PICK1 complexes may have a defined molecular composition in these cells, determining their intracellular targeting and clustering. Consistent with this, α7 clusters in populations of spinal cord neurons differently colocalize with cytoskeletal and lipid rafts components indicating that α7-containing protein complexes can be different between neuron populations (Roth and Berg, 2003).

Our data introduce PICK1 as first intracellular protein that controls clustering of nAChRs in the CNS, exemplified by α7 nAChRs. Since PICK1 does not interact with nAChR subunits α4 and β2 in our tests, PICK1’s effects may be specific for the α7 receptors within the family of all nAChRs. Very little is known about clustering mechanisms for other nAChRs in the peripheral and central nervous system, while many players are known that regulate synaptic aggregation of muscle AChRs at the neuromuscular junction, as reviewed recently (Wiesner and Fuhrer, 2006). In chick ciliary ganglion, clustering of heteromeric nAChRs (α3, α5, β2 and β4 subunits) depends on signals within the cytoplasmic loop of α3 and requires postsynaptic functioning of APC protein (Temburni et al., 2004; Williams et al., 1998). PSD-93 and PSD-95 associate with the nAChRs and form a scaffold for nicotinic signaling (Conroy et al., 2003). In rodent superior cervical ganglion, formation and stabilization of cholinergic interneural synapses (containing clustered heteromeric nAChRs) require agrin and PSD-93, respectively (Gingras et al., 2002; Parker et al., 2004). At the neuromuscular junction, agrin/MuSK signaling and many intermediate proteins direct synaptic formation and AChR clustering (reviewed by Strochlic et al., 2005), APC being one requirement for AChR clustering (Wang et al., 2003), and rapsyn acting as an anchor (Gautam et al., 1995).

Possible mechanisms and relevance of PICK1 controlling α7 clustering
The pronounced reduction in surface α7 clustering by PICK1 in interneurons implies that not only receptors at GABAergic synapses are affected but also clusters that most likely represent extrasynaptic receptor aggregates. Regulation by PICK1 thus appears as a common property of all α7 receptor clusters in these cell cultures. There are many possibilities by which PICK1 could reduce α7 clustering. PICK1 could disperse surface receptor aggregates leading to diffuse receptors undetectable by our staining. In addition, PICK1 may reduce delivery of newly
synthesized α7 nAChRs to the plasma membrane, or promote receptor internalization. The actions of PICK1 on other neurotransmitter receptors, together with the known protein interactions of PICK1 (Jin et al., 2006; Perez et al., 2001; Takeya et al., 2000), are compatible with any or even a combination of these possibilities. Functional expression of α-BT-binding α7 nAChRs is also regulated by palmitoylation of α7 receptors during their assembly in the ER (Drisdel et al., 2004), and tyrosine dephosphorylation can increase levels of α7 receptor at the surface (Cho et al., 2005), although clustering is not affected (Charpantier et al., 2005).

The neuronal network in the CNS is vulnerable to calcium-induced excitotoxicity, raising the need for control of calcium influx into individual neurons. Due to the fact that the α7 nAChR is highly permeable to calcium ions and involved in neuronal survival (Dajas-Bailador and Wonnacott, 2004; Seguela et al., 1993), the activity, distribution and clustering of this receptor should be precisely controlled. Our results have strong implications for PICK1 to play a role in these processes. Furthermore, controlling α7 clustering on hippocampal GABAergic interneurons could allow PICK1 to control the disinhibition of pyramidal cells in LTP, providing a potential mechanism for the role of α7 in learning, as the activity of postsynaptic α7 receptors on GABAergic interneurons influences hippocampal inhibition (Alkondon et al., 1997; Jones and Yakel, 1997), and as activation of these receptors blocks concurrent STP and LTP induction in pyramidal cells innervated by these interneurons (Ji et al., 2001). Finally, postsynaptic α7 nAChRs on GABAergic interneurons are also important in hippocampal sensory gating (Martin et al., 2004). Auditory gating is diminished with schizophrenia and used as a model for this disease in rodents (Martin et al., 2004; Ripoll et al., 2004). Interestingly, PICK1 polymorphism is associated with schizophrenia (Hong et al., 2004) and recent data implicate PICK1 as a susceptibility gene for schizophrenia (Fujii et al., 2006), while on the other hand many genetic and other studies have linked α7 to this disease (Ripoll et al., 2004).

In summary, modulation of α7 nAChR activity and clustering may form one aspect of the various emerging roles of α7 nAChRs ranging from synaptic to systems level, including neuronal survival, nicotine addiction, synaptic plasticity in learning, and neurological disease. While recent progress has identified phosphorylation mechanisms as regulators of α7 nAChR activity (Charpantier et al., 2005; Cho et al., 2005), the present report implies PICK1 to control α7 nAChR clustering in the brain. The intracellular mechanisms and the relevance of this control for α7-mediated physiological and pathological processes remain to be investigated.
Experimental Methods

Identification and cloning of PICK1 and yeast two-hybrid assay. Yeast two-hybrid (YTH) screening (Fields and Song, 1989) was performed using the Matchmaker System 3 (Clontech, Palo Alto, California) according to the manufacturer’s protocol, in order to identify α7 nAChR-binding proteins. The cytoplasmic loop of rat α7 nAChR cDNA (amino acids 332-467; α7 cDNA was a gift from Dr. Jim Boulter, UCLA, California) was inserted in-frame into the pGBKT7 bait vector. A rat brain cDNA library in vector pACT2 (Clontech) was used. Yeast cells (AH109) were sequentially cotransformed with α7 bait and library prey vectors, and then plated on selection medium lacking Ade, Trp, Leu and His. Two independent full-length PICK1 clones expressing His3, Ade and β-galactosidase activity were isolated. Positive clones were cotransformed with the bait vector or control plasmids into the AH109 yeast strain to confirm the interaction (i) on selection plates, (ii) with the Gal lift filter assay and (iii) using X-α-Gal indicator plates according to the manufacturer’s instructions (Clontech). All bait and prey plasmids used were from PCR products subcloned in frame into pGBK7, pACT2 or pGADT7 vectors and were confirmed by DNA sequencing. The α7 nAChR bait sequences were PCR amplified and inserted into the bait vector using EcoRI and BamHI restriction sites. For example, the following primer pairs were used for α7 nAChR bait plasmid construction: bait 1 (aa 332-467) 5’-GCGCGGAATTCAGAATCATTCTCCTGAAC + 5’-GCGCGGATCCTCACACCACGCAGGCTGC; bait 9 (aa 429-467) 5’-GCGCGGAATTCGGGGACCCCGACCTGGCC + 5’-GCGCGGATCCTCACACCACGCAGGCTGC; bait 10 (aa 371-467) 5’-GCGCGGAATTCCTGAGTGCGTGCTGGG + 5’-GCGCGGATCCTCACACCACGCAGGCTGC. The PICK1 prey sequences were PCR amplified and inserted into the prey vector using EcoRI and BamHI restriction sites. Other information for cDNA constructs is indicated in the Figures. To verify protein expression of bait constructs, yeast protein extracts were prepared of transformed yeast cells using the Urea/SDS method (Clontech) and analysed by Western blotting with the anti-GAL4 DNA-BD monoclonal antibody (Clontech)(data not shown). The point mutations in the cytoplasmic loop of rat α7 nAChR were introduced using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer pair to change the motif EVRY to EARA (aa 439-442) was 5’-TCCTGGAGGAGGCCCGCCGGCCATCGCCAACCACGC + 5’-GCGGTTGGCAGATGGCGCGGGCCTCCTCCAGGA. The primer pair to change the motif ESEV
to EAEA (aa 452-455) was 5’- CTGCCAGGACGAGGCTGAGGCGATCTGCAGTGAATGG + 5’- CCATTCACTGCAGATCGCCTCAGCCTCGTCCTGGCAG. Constructs were verified by sequencing. The NCBI accession numbers are AF327562 for rat PICK1 and L31619 for rat α7 nAChR.

**Other DNA constructs.** C-terminally Flag-tagged mouse α7 nAChR in pCS2+ expression vector was a gift from Dr. Ines Ibanez-Tallon (MDC Berlin-Buch). N-terminally EYFP-tagged rat PICK1 in pRK5 expression vector (referred to as PICK1-EYFP) and the empty control EYFP vector were a gift from Prof. Ann Marie Craig and Dr. Fernanda Laezza (Washington University). All other constructs were generated according to standard molecular biology techniques.

**Fusion proteins, bacteria, COS-7 transfection and in-vitro binding.** Full-length rat PICK1 or the cytoplasmic loop of rat α7 nAChR (aa 319-467) or of α4 nAChR were subcloned in frame into the GST-fusion vector pGEX-2T (Pharmacia, Piscataway, New Jersey). PICK1 was also subcloned into pET-28a(+) vector (carrying a His-tag; Novagen, EMD Biosciences, Darmstadt, Germany); and PICK1 was myc-tagged and cloned into pcDNA3 expression vector (Invitrogen). All constructs were confirmed by sequencing. The *E. coli* strain DH5α was used to express GST fusion proteins and the strain BL21 to express His-PICK1, in both cases using IPTG as an inducer. GST fusions were purified using glutathione-sepharose beads as described previously (Fuhrer and Hall, 1996). Bacteria were transfected using a standard heat shock procedure. COS-7 cells were transfected with α7 or myc-PICK1 constructs using Fugene 6 Transfection Reagent (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, IN). Transfected bacteria and COS cells were lysed in bacterial (Smith and Johnson, 1988) and eucaryotic (Fuhrer et al., 1997) cell lysis buffer, respectively, and incubated with purified fusion proteins, i.e. GST-PICK1, GST-α7loop, or GST-α4loop immobilized to beads. Beads were pelleted, washed with lysis buffer, and analyzed by α7-, myc- or His-immunoblotting. Blots were reprobed for GST. For detection, mouse monoclonal anti-myc antibodies (Sigma), goat polyclonal anti-α7 antibodies (Drisdel and Green, 2000)(Santa Cruz Biotechnology), mAb306 (against α7)(Rangwala et al., 1997; Schoepfer et al., 1990), mouse monoclonal anti-GST antibodies (Santa Cruz Biotechnology), and rabbit polyclonal anti-T7 tag antibodies (His-tag; Novagen) were used.

**Rat brain preparation and α7 precipitation.** Synaptosomes were prepared from dissected adult rat hippocampus as described previously (Carlin et al., 1980). Briefly, tissue was homogenized in
buffer A (0.32 M Sucrose, 1 mM NaHCO$_3$, 1 mM MgCl$_2$, 0.5 mM CaCl$_2$) on ice and centrifuged at 1400 g for 10 min at 4°C, and the supernatant was saved (S1). The pellet (P1) was resuspended in buffer A, centrifuged at 720 g for 10 min at 4°C and the pellet (P2) discarded. S2 and S1 were combined, centrifuged at 720 g for 10 min at 4°C and pellets (P3) were discarded. The supernatants S3 were centrifuged at 13,800 g for 10 min at 4°C and the supernatant (S4) discarded. The pellet (P4) was resuspended in buffer B (50 mM Tris, 150 mM NaCl, 5 mM EDTA; pH 7.4; containing protease inhibitors (Complete Mini protease inhibitor tablets; Roche, Switzerland), and 0.5 % Triton X-100 was added. Samples were rotated for 15 min at 4°C and split into two identical portions. To one sample, free α-BT was added (10 μM final concentration) and both samples were rotated for 60 min at 4°C.

Alternatively, lysates from adult rat brain membranes were prepared as described previously (Chen and Patrick, 1997). Briefly, brain tissue was homogenized in buffer A1 (50 mM sodium phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride) on ice, centrifuged 2 times at 100’000 g for 1 h at 4°C, and the supernatants were discarded. The pellet was resuspended in ice-cold buffer A2 (buffer A1 plus 2% Triton X-100 and protease inhibitors), rotated for 2 h at 4°C, and centrifuged at 100’000 g for 1 h at 4°C. The supernatant was split into two identical samples to which 10 mM nicotine and vehicle, respectively, were added. Samples were rotated for 1 h at 4°C.

For precipitation with α-BT, 50 μl of α-BT coupled to sepharose beads (Fuhrer and Hall, 1996) were added to both samples (prepared either from hippocampal synaptosomes or from whole brain membranes, see above) for 2 h. Alternatively, for the α7 immunoprecipitation, 1 μl of anti-α7 nAChR antibody mAb319 (Sigma)(Rangwala et al., 1997; Schoepfer et al., 1990) was added for 1 h, followed by Protein G-Sepharose (Amersham Biosciences AB, Uppsala, Sweden). In controls, mAb319 was replaced by an identical amount of rat non-immune IgG. Beads were pelleted, washed with buffer B, and proteins eluted with Lammli buffer at 80°C and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes for Western blotting. The anti-PICK1 antibody (Upstate Biotech, New York) was used at 1:500, the anti-α7 antibody (mAb306, mAb319; or ab10096 from Abcam Ltd., Cambridge UK (Charpantier et al., 2005)) at 1:1000, the anti-PSD95 family antibody (Upstate) at 1:1000, and the anti-GluR2 antibody (Chemicon, MAB397) at 1:1000. Rat brain microsomal preparations (Upstate) were used as positive controls for the PICK1 signal according to the manufacturer’s instructions (data not shown). HRP-conjugated secondary antibodies (Zymed) were used and detected with enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate kit (Pierce)).
Cell culture and immunocytochemistry of primary hippocampal neurons. Rat embryos were obtained from time-pregnant Wistar rats (RCC Laboratory Animal Services, Füllinsdorf, Switzerland). All experiments were approved by the cantonal veterinary office of Zürich. Primary cultures of embryonic day (E)18-19 hippocampal neurons were prepared as described previously (Brunig et al., 2002). The cells were grown in neurobasal medium (Gibco) supplemented with B27 supplement (Gibco), 0.5 mM L-glutamine, and 1.25 mg/ml gentamicin (Gibco) in the presence of a glial feeder cell layer. The cells were plated at 1.5 x 10⁴ per 18 mm glass coverslip previously coated with poly-L-lysine (Sigma) and used for immunocytochemistry after 2-3 weeks.

For the magnetofection method, hippocampal neurons were cultivated as described (Chudotvorova et al., 2005) at a density of 5 x 10⁵ cells per coverslip for 11 days in 5% CO₂ and 37°C in the absence of a glial feeder cell layer in MEM medium (Invitrogen) containing 15% NU serum (BD), 2% B27 supplement, 0.015 M HEPES pH 7.1, 0.45% glucose, 1 mM sodium pyruvate (Invitrogen), and 2 mM L-Glutamine (Gibco).

In all cases, immunocytochemistry was performed according to Brunig et al. (2002a). In brief, the living cultures were incubated for 30-60 min at room temperature with 100 nM α-BT coupled to rhodamine, Alexa 488 or Alexa 647 (Molecular Probes) in medium or Ringer’s solution (in mM: CaCl₂ 2, MgCl₂ 2, glycine 0.001, with or without TTX 0.0005, glucose 30, HEPES 25, KCl 5, NaCl 119, pH 7.4) (Archibald et al., 1998). They were subsequently washed with Ringer’s solution and fixed with 4% PFA in 0.15 M phosphate buffer for 15 min at RT, followed by washing with PBS and permeabilisation for 5 min at RT using 0.2% Triton-X 100 in PBS containing 10% normal goat serum (NGS). Fixed cultures were rinsed extensively with PBS and incubated for 90 min at RT with the following antibodies diluted in PBS containing 10% NGS: rabbit immunoaffinity purified anti-PICK1 (Upstate Biotech., diluted 1:50), rabbit polyclonal or mouse monoclonal anti-VGAT (Synaptic Systems, diluted 1:1000 or 1:500, respectively), rabbit polyclonal anti-GAD65/67 (Affinity, diluted 1:2000), mouse monoclonal anti-bassoon (Stressgen Bioreagents, Ann Arbor, Michigan, 48108 USA, diluted 1:500), mouse monoclonal anti-gephyrin (mAb7a, Connex, Martinsried, Germany, diluted 1:800), or rabbit polyclonal anti-PSD-95 (Cho et al., 1992) (diluted 1:1000). The mouse monoclonal anti-GluR2 antibody against the large N-terminal extracellular domain of GluR2 (Chemicon, diluted 1:200) was incubated on living cultures as described above. Cultures were subsequently washed with PBS and incubated with secondary antibody coupled to Alexa 488, Alexa 350, or rhodamine (Molecular Probes, Jackson Laboratories, Füllinsdorf, Switzerland).
Laboratories; diluted 1:200) for 30 min at RT in PBS plus 10% NGS. After washing in PBS, cells were mounted in Mowiol and stored at 4°C.

**COS-7, HEK 293T and SH-SY5Y cells, neuron transfection and staining.** Cells were plated onto glass coverslips. COS-7, HEK 293T and SH-SY5Y cells were used 48 h after transfection or electroporation, and neurons were analysed 24 h after magnetofection. COS-7 cells were transfected with HA-tagged PICK1 and nAChR α7 expression constructs using the Fugene transfection reagent (Roche, Indianapolis, IN) according to the manufacturer’s instructions. HEK 293T cells and SH-SY5Y cells stably overexpressing nAChR α7 subunit (Charpantier et al., 2005) were electroporated with different constructs using the nucleofection method according to manufacturer’s instructions (Amaxa Biosystems, Cologne, Germany). Hippocampal neurons were transfected using the magnetofection method at 11 days in vitro with CombiMag (OZ Biosciences) as described earlier (Chudotvorova et al., 2005). The following plasmids were used: PICK1-EYFP, EYFP, and/or Flag-tagged α7 constructs. Living SH-SY5Y cells and neurons were incubated for 60 min with α-BT coupled to rhodamine (Molecular Probes, 100 nM) and/or with a mouse monoclonal anti-Flag antibody (Sigma, diluted 1:1000), and/or with a rabbit polyclonal anti-GABA<sub>A</sub> receptor α1 subunit antibody (Fritschy and Mohler, 1995)(diluted 1:5000), and subsequently washed with PBS. All cells were fixed with 4% PFA, permeabilized and stained as described above for neuronal cells using the following primary antibodies: mouse (Roche, diluted 1:1000) or rat monoclonal anti-HA (Roche, diluted 1:200), mAb306 (diluted 1:200), or rabbit polyclonal anti-α7 (ab10096 from Abcam Ltd., diluted 1:200).

**Viral infection.** Neurons were used after 14 d in vitro, transferred to a dish containing conditioned medium without the glia feeder cell layer and were incubated with or without Sindbis virus. We used the same Sindbis constructs and conditions as previously described (Terashima et al., 2004). Incubation was done for 17-22 h (Perez et al., 2001; Terashima et al., 2004), after which cells were incubated with α-BT-rhodamine, washed, fixed and analyzed by epifluorescence or confocal microscopy.

**Data analysis.** Experiments were analyzed by epifluorescence microscopy using a high-resolution digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) or by confocal laser scanning microscopy (TCS 4D; Leica, Deerfield, IL). Images were acquired with a 100x lens (numerical aperture 1.4) at a magnification of 0.11 μm/pixel. Controls in which one or more primary
antibodies were omitted indicated no significant cross-contamination among fluorescence channels. Imaging conditions were kept constant for each channel. Contrast-optimized images using the Photoshop software were analyzed with the ImageJ imaging software (NIH) keeping constant threshold levels. Clusters were defined by their intensity (more than twice the intensity of the surrounding membrane) and size (at least 9 adjacent pixels).

Quantitative analyses after virus infection (Fig. 7) were performed on randomly selected samples in a total of 121 cells originating from three independent cultures (9 GFP virus-infected cells; 43 non-infected cells; 29 WT virus-infected cells; 40 AA virus-infected cells). Along three membrane regions of each cell (soma, proximal and distal dendrites), four areas, each covering 100 μm², were randomly chosen per region. The boxes in Figure 7 show examples of somatic areas. Definitions were: proximal dendrites, dendritic areas from the soma to a distance of about 140 μm; distal dendrites, dendritic areas on smaller branching dendrites further away than 140 μm from the soma. In Figure 8, segments of 75 μm² were selected on proximal dendrites from 86 cells from 2 independent cultures (30 nontransfected cells, 31 EYFP transfected cells, 25 PICK1-EYFP transfected cells). Within each area, the surface covered by α-BT-fluorescence was measured with the ImageJ imaging software (NIH), and values were expressed as mean +/- SEM.

To quantitate the number of α7 nAChR clusters and their colocalization with PICK1, we randomly selected segments of 100 μm dendrite length (145 segments from 40 cells of three independent cultures). Clusters of α7 and PICK1 were counted in these segments and expressed as number of α7 clusters or percentage of colocalization of PICK1 with α7 clusters.

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Figure legends

Figure 1. Interaction between α7 nAChR and PICK1 in yeast.
(A) Yeast strain AH109 was cotransformed with plasmids encoding the GAL4 DNA-binding domain fused to different sequences of the cytoplasmic loop of rat α7 nAChR (or rat α4 or β2 nAChR, as indicated) and the GAL4 activation domain fused to different PICK1 sequences. Protein-protein interaction was assayed by growing the yeast on selective medium and by galactosidase assays. The specificity of this interaction was tested using control plasmids (not shown). + indicates interaction, - no interaction. n.d., not done. (B) PICK1 prey constructs used. CC, coiled coil domain; AR, acidic region. (C) Mutation of C-terminal putative PDZ-binding motifs in α7 nAChR bait 1 and bait 9. The interaction with PICK1 prey vectors was not affected.

Figure 2. Interaction of recombinant α7 and PICK1.
(A, B and C) COS cells were transfected with full-length α7 or myc-PICK1 expression constructs, lysed and incubated with the indicated amounts of GST proteins immobilized on beads. Bead pellets were analysed by α7- or myc-immunoblotting, and blots were reprobed for GST, showing that GST-PICK1 precipitates α7 from the COS lysate (A), while GST-α7loop pulls down myc-PICK1 (B), and GST-α4loop does not pull down myc-PICK1 (C). As a control, non-transfected COS cells produced no immunoblot signals (not shown). Panels of GST-blots show GST, GST-α7loop or GST-α4loop proteins at their respective molecular weights. To probe α7 nAChR, the following antibodies were used for immunoblots: polyclonal anti-α7 (Santa Cruz; shown) and mAb306 (not shown), with identical results. (D) Bacteria expressing His-PICK1 were lysed, incubated with the indicated GST beads, and precipitates were analyzed by His-immunoblotting, showing that GST-α7 loop pulls down His-PICK1. Parallel samples were Coomassie-stained to reveal GST and GST-α7 loop proteins, shown at their respective molecular weight.

Figure 3. Interaction of endogenous α7 nAChRs and PICK1 in adult rat brain.
(A) Synaptosomes were prepared from dissected hippocampi of adult rats, and α7 nAChRs were precipitated with α-BT-Sepharose beads (Tox-P). As controls for specificity, excess free α-BT (+T) was added. A fraction of the total synaptosomal lysate was loaded as a control (Tot). PICK1 immunoblotting reveals specific association with α7 nAChRs, which themselves are visualized in
an α7-blot using mAb306 (shown) or mAb319 (not shown; identical results). (B) From adult rat brain lysates, α7 nAChRs were precipitated with α-BT-Sepharose beads (Tox-P) and analyzed by PICK1- or α7-immunoblotting (anti-α7 from Abcam). Nicotine-competition eliminated the α7 nAChR signal and strongly reduced the PICK1 signal, demonstrating specific α7-PICK1-association. (C) Synaptosomes (left) or total hippocampal tissue (right) were prepared from hippocampus (Hip), cerebellum (Cer) or cortex (Cor), lysed, and α7 precipitated using mAb319. As controls, mAb319 was omitted (Ab), brain tissue was left out, or a fraction of total hippocampal synaptosomes was loaded without precipitation (Tot). α7-associated PICK1 was visualized by immunoblotting and mostly detected in hippocampus. Levels of α7 were highest in hippocampus, as revealed by α7-immunoblotting (not shown). Nonimmune IgG was used as a control (right). *indicates the antibody band. (D) Hippocampal synaptosomes were processed as in C, but antibodies against the PSD95-family or GluR2 were used for immunoblotting. PSD95-family proteins and GluR2 AMPAR subunits were present in hippocampal synaptosomes but not associated with α7.

Figure 4. Partial colocalization of α7 and PICK1 in transfected heterologous cells. (A) COS cells were transfected either with α7 expression vector or with HA-tagged PICK1 expression construct (a-c). Alternatively, they were transfected with both plasmids (d-f). Cells were permeabilized, stained for α7 using anti-α7 antibodies (red), HA-tag (green), or both, and analyzed by conventional fluorescence microscopy. Anti-α7 antibodies were from Abcam (shown) or mAb306 (not shown), with identical results. In all cases, α7 and PICK1 signals are diffuse and around the nucleus. Coexpression does not affect this and reveals partial overlay in the perinuclear area (yellow). Untransfected COS cells produced no signal (not shown). Scale bar, 20 μm. (B) HEK 293T cells were transfected with α7-Flag and PICK-EYFP constructs and stained using antibodies against the Flag-tag (red). Confocal analysis reveals diffuse α7 and PICK1 signals and clusters of PICK1 (b). The diffuse pattern, but not the PICK1 clusters, shows some overlap between the two proteins (yellow in c). The distribution of α7 and PICK1 was identical in singly transfected cells (not shown). Scale bar, 10 μm. (C) SH-SY5Y cells stably expressing α7 were electroporated with PICK1-EYFP expression vector. They were subjected to surface staining of α7 (using α-BT-Alexa 647, red) and analyzed by fluorescence microscopy. Two cells expressing α7 clusters are visible in a, and only the upper cell expresses PICK1-EYFP (b). This EYFP signal (green) is diffuse and in clusters, which partially overlap with the α7 clusters (yellow in c). In the
lower cell, and in cells not transfected with PICK1-EYFP (not shown), α7 clusters are very similar. (D) As control for specificity, SH-SY5Y cells were stained with rhodamine-α-BT in the presence or absence of nicotine. Phase contrast (PC) shows the cells present. Nicotine-competition (1 mM nicotine added 10 min before rhodamine-α-BT) caused a strong reduction in α-BT surface staining, demonstrating the specificity of the α-BT signal for α7 clusters. Scale bar, 20 μm.

**Figure 5. α7 nAChRs are clustered at the surface of GABAergic hippocampal interneurons.**

Cultured dissociated hippocampal neurons were labeled with fluorescent α-BT, permeabilized, and incubated with antibodies against VGAT or GAD followed by fluorescent secondary antibodies. Image overlays reveal that α7-expressing cells are VGAT- and GAD-positive. α7 clusters occur on the soma, often grouped into larger aggregates, and along dendrites, and show some overlap with VGAT or GAD. Panels a-c show a maximal projection of confocal stacks, while panels d-f represent a single confocal section. Scale bars: 20 μm.

**Figure 6. Colocalization of α7 and PICK1 in clusters at the surface of hippocampal interneurons.**

Cultured hippocampal neurons were labeled with rhodamine-α-BT, permeabilized, and incubated with PICK1 antibodies, followed by secondary Alexa 488-coupled antibodies and conventional fluorescence microscopical analysis. Panels a-c and d-f represent two different fields at slightly different magnification. Clusters of α7 partially colocalize with clusters of PICK1 (yellow in overlay, and some examples highlighted by arrows in a-c). Clusters of PICK1 are not only seen on α7-positive interneurons, but also on α7-negative cells that most likely represent pyramidal neurons (white boxes in b and c). Scale bars: 10 μm.

**Figure 7. Viral expression of PICK1 causes a reduction in surface α7 nAChR clusters in cultured hippocampal interneurons.**

Cultured hippocampal cells were infected with different Sindbis viruses (SV), labeled with α-BT-rhodamine and analyzed by conventional fluorescence microscopy. The panels show examples of EGFP fluorescence (right) and surface α7 nAChR staining by α-BT-rhodamine for a non-infected control neuron, a neuron infected with SV containing EGFP, a neuron infected with SV expressing PICK1-WT and EGFP, and a neuron infected with SV containing PICK1-AA mutant and EGFP. Inserts show magnifications of α-BT-staining of somatic regions indicated by the box (for lower-
power images, scale bars are 20 μm). Wild-type PICK1 expression reduces α7 clustering. A quantitative analysis of these effects is shown at the bottom. For each neuron in a group (non-infected, EGFP virus, PICK1-WT-EGFP virus, or PICK1-AA-EGFP virus), four surface areas covering 100 μm² (comparable to the boxes indicated in the top panels) were randomly chosen per cellular region (s, soma; p, proximal dendrites). The α-BT fluorescence intensity was quantitated and plotted per surface area (***p<0.0001; **p<0.0015, by unpaired two-tailed Student’s t-test). α7 surface clustering is reduced by PICK1-WT but not by PICK1-AA in somatic and proximal dendritic areas.

Figure 8. Expression of PICK1 by magnetofection causes a reduction in surface α7 nAChR clusters in cultured hippocampal interneurons.
Cultured hippocampal cells were transfected with PICK1-EYFP or EYFP constructs using magnetofection, labeled with α-BT-rhodamine and anti-VGAT antibody and analyzed by conventional fluorescence microscopy. The panels show examples of surface α7 nAChR staining by α-BT-rhodamine (left), EYFP fluorescence (middle), and VGAT staining (right) for a non-transfected control interneuron, an interneuron transfected with EYFP, and an interneuron transfected with PICK1-EYFP. PICK1 expression reduces α7 clustering. A quantitative analysis of these effects is shown at the bottom. For each neuron in a group (untransfected, PICK1-EYFP transfected or EYFP transfected), proximal dendritic surface areas were randomly chosen (the boxes represent examples). The amount of α7 surface clusters on dendrites of transfected neurons was measured as the cumulative α-BT fluorescence area per dendritic surface area. α7 surface clustering is reduced by PICK1-EYFP but not by EYFP in dendritic areas (*p=0.0267; unpaired two-tailed Student’s t-test). Scale bar, 20 μm.

Figure 9. Expression of PICK1-EYFP does not affect the distribution of GABA_A receptors at the surface of interneurons.
PICK1-EYFP or EYFP constructs were expressed in cultured hippocampal neurons using magnetofection. Neurons were stained against GABA_A receptor α1 subunit (red; before permeabilization) and with VGAT-antibodies (blue; after permeabilization) as markers for interneurons. The panel shows two representative interneurons, overexpressing PICK1-EYFP (left) or EYFP (right). The four small images on the top show the PICK1-EYFP or EYFP signal, phase contrast (PC), VGAT signal and the merged image. The large images below show the GABA_A receptor α1 subunit signal. Note the abundant GABA_A receptor surface clusters in both
interneurons and the healthy morphology of the cells. The GABA<sub>\alpha</sub>1 receptor signal is not affected in interneurons after PICK1-EYFP expression compared to control EYFP expression. Scale bars, 20 μm.
Figure 1

A

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class I | class II | class III | mutated
Figure 2

A) COS lysate: α7

Beads: GST, GST, GST-PICK1

μg: 8, 2, 2

Pulldown / α7-blots: 47–

GST-blots: 24–77

B) COS lysate: myc-PICK1

Beads: GST, GST, GST-α7loop

μg: 8, 2, 8

Pulldown / myc-blots: 52–

GST-blots: 24–35

C) COS lysate: myc-PICK1

Beads: GST, GST-α4loop

μg: 8, 8

Pulldown / myc-blots: 52–

GST-blots: 24–35

D) Bact. lysate: His-PICK1

Beads: GST, GST, GST-α7loop

μg: 10, 2, 2

Pulldown / His-blots: 47–

His-PICK1: 24–35
Figure 7

α-BT

no SV

SV-EGFP

SV-PICK1-WT-EGFP

SV-PICK1-AA-EGFP

EGFP

α-BT signal/100 µm²

non-inf s 
GFP s 
WT s 
AA s 
non-inf p 
GFP p 
WT p 
AA p