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Presynaptic PICK1 facilitates trafficking of AMPA-receptors between active zone and synaptic vesicle pool

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Abstract

Previous studies have indicated that presynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPARs) contribute to the regulation of neurotransmitter release. In hippocampal synapses, the presynaptic surface expression of several AMPAR subunits, including GluA2, is regulated in a ligand dependent manner. However, the molecular mechanisms underlying the presynaptic trafficking of AMPARs are still unknown. Here, using bright-field immunocytochemistry, western blots, and quantitative immunogold electron microscopy of the hippocampal CA1 area from intact adult rat brain, we demonstrate the association of AMPA receptors with the presynaptic active zone and with small presynaptic vesicles, in Schaffer collateral synapses in CA1 of the hippocampus. Furthermore, we show that GluA2 and protein interacting with C kinase 1 (PICK1) are colocalized at presynaptic vesicles. Similar to postsynaptic mechanisms, overexpression of either PICK1 or pep2m, which inhibit the N-ethylmaleimide sensitive fusion protein (NSF)-GluA2 interaction, decreases the concentration of GluA2 in the presynaptic active zone membrane. These data suggest that the interacting proteins PICK1 and NSF act as regulators of presynaptic GluA2-containing AMPAR trafficking between the active zone and a vesicle pool that may provide the basis of presynaptic components of synaptic plasticity.

Keywords: Synaptic plasticity, hippocampus, electron microscopy, AMPA, PICK1, receptor trafficking.

Introduction

The majority of fast excitatory glutamatergic neurotransmission in the CNS is mediated by AMPARs that underpin cognitive processes like learning and memory (Derkach et al., 2007; Anggono and Huganir, 2012). Furthermore, AMPAR dysfunction underlies neurodegenerative diseases such as stroke and epilepsy (De Sarro et al., 2005; Kwak and Weiss, 2006; Bowie, 2008). AMPARs are assembled from subunits GluA1-4 into functional homo- or hetero-tetrameric cationic channels that are permeable to sodium and calcium (Hollmann and Heinemann, 1994; Bettler and Mulle, 1995; Traynelis et al., 2010). In the hippocampus, the majority of the AMPARs consist of the heteromers GluA1-GluA2 and GluA2-GluA3 (Wenthold et al., 1996). At the excitatory Schaffer collateral/commissural synapses onto CA1 hippocampal pyramidal cells, about 80 % of postsynaptic AMPARs and >95 % of extrasynaptic AMPARs are GluA1-GluA2 heteromers, and most of the remaining receptors are GluA2-GluA3 heteromers (Lu et al., 2009). Thus, virtually all functional postsynaptic AMPARs in these synapses contain GluA2.

The postsynaptic concentration of AMPARs is controlled by the equilibrium between insertion, removal and lateral diffusion of the plasma membrane receptors. These are highly regulated processes dependent on neuronal activity (Henley and Wilkinson, 2016). Several synaptic proteins are involved in various steps of AMPAR regulation and trafficking, including PICK1, stargazin, postsynaptic density protein of 95 kDa (PSD-95), glutamate receptor interacting protein 1 (GRIP1), AMPAR binding protein (ABP), protein kinase C (PKC), and N-ethylmaleimide-sensitive factor (NSF) (Henley and Wilkinson, 2013).

Although postsynaptic AMPARs are extensively studied neuronal proteins, the localization, trafficking and functions of presynaptic AMPARs is poorly understood. However, we and others have previously suggested that presynaptic AMPARs may function as autoreceptors, regulating neurotransmitter release (Patel and Croucher, 1997; Schenk et al., 2003; Feligioni et al., 2006; Pittaluga et al., 2006; Haglerød et al., 2009; Summa et al., 2011; Grilli et al., 2012). Intriguingly, we have also demonstrated that presynaptic AMPARs appear to be regulated in an opposite direction to the

postsynaptic receptors by AMPA and NMDA stimulation (Feligioni et al., 2006). Given the comparative paucity of information about presynaptic AMPARs we investigated if the same regulatory proteins that control postsynaptic AMPAR trafficking are also responsible for presynaptic AMPAR regulation. In particular we focused on PICK1 and NSF, both of which have well-characterized roles in the control of postsynaptic AMPARs.

PICK1 interacts with both GluA2 and PKC at the postsynaptic site to regulate GluA2 phosphorylation, thus altering its synaptic clustering, trafficking to neuronal surface and membrane recycling (Hastie, 2009). The interaction between PICK1 and GluA2 is involved in the removal of GluA2 from the plasma membrane during long-term depression (LTD) in the hippocampus (Iwakura et al., 2001; Kim et al., 2001; Hanley and Henley, 2005) and the cerebellum (Xia et al., 2000; Steinberg et al., 2006; Hanley, 2008; Terashima et al., 2008).

NSF is an ATPase that is essential to provide the energy for presynaptic vesicular fusion events (Sollner et al., 1993) (Sudhof and Rizo, 2011). Intriguingly, NSF also plays a vital role at the postsynaptic membrane and is necessary for plasma membrane insertion of GluA2-containing AMPARs, increasing its surface expression (Huang et al., 2005; Araki et al., 2010). NSF binds the GluA2 C-terminus upstream of the PDZ binding site between residues Lys844 and Gln853. Disrupting NSF binding to GluA2 decreases AMPAR mediated synaptic transmission (Nishimune et al., 1998; Song et al., 1998) and the surface expression of GluA2 (Noel et al., 1999). One way of impeding the NSF/GluA2 interaction is by introducing a peptide (pep2m) corresponding to the interaction site on GluA2 (Nishimune et al., 1998; Collingridge and Isaac, 2003; Evers et al., 2010).

We have shown previously that PICK1 is present in the presynaptic terminal at concentrations comparable to those seen in postsynaptic spines (Haglerød et al., 2009). Inhibition of the interaction between PICK1 and GluA2 in rat hippocampal and cortical synaptosomal preparations alters AMPAR-mediated nor-adrenaline release (Pittaluga et al., 2006). Moreover, in the nucleus accumbens, presynaptic AMPARs have been reported to modulate nerve terminal dopamine release (Grilli et al., 2012).

To explore the relationship between presynaptic AMPARs and PICK1, we used western blotting and immunocytochemistry at light and electron microscopical levels to determine whether both proteins were present in the same neurons and in the same presynaptic terminals. We then used viral transduction of PICK1 or pep2m, a peptide that selectively inhibits NSF-GluA2 interactions, to determine the effects of increasing the presynaptic concentration of PICK1, or impeding the GluA2-NSF interaction, on the surface expression of GluA2.

Our data provide evidence that PICK1 and NSF can act to regulate presynaptic trafficking of GluA2-containing AMPARs between a vesicle pool and the active zone.

Experimental procedures

Antibodies

The following primary antibodies were used: Anti-PICK1 (Abcam, Cambridge, UK, Cat# ab3420) was used at 1:10-1:15 for electron microscopy (EM) and 1:75 for light microscopy (LM). Anti-PICK1 (C100, from Jun Xia, Hong Kong) was used at 1:1,500 for western blot (WB). Anti-GluA2 (Lifespan, Seattle, USA, Cat # LS-B1473) was used at 1:5,000 for WB. Anti-GluA2 (Alomone labs, Jerusalem, Israel, Cat# AGC-005) was used at 1:75 for LM, and at 1:40 for EM. Anti-GluA2/3 (Millipore, MA, USA, Cat# AB1506) was used at 1:30 for electron microscopy (EM). Anti-ß-tubulin (Tuj1) (Covance, CA, USA, MMS-435P) was used at 1:10,000 for WB. *The following secondary antibodies were used:* IgG coupled to 10 nm and 20 nm colloidal gold (British BioCell International, Cardiff, UK, Cat#R14007) was used at 1:20-1:40 for EM. Goat anti-rabbit alkaline phosphatase (Sigma, MO, USA, Cat#A3687) was used at 1:10,000 for WB. Biotinylated goat anti-rabbit (Abcam, Cambridge, UK, Cat#Ab64256) was used at 1:100 together with a streptavidin biotinylated horseradish peroxidase complex (GE healthcare, Buckinghamshire, UK, Cat#RPN1051V) at 1:100 for light (LM).

The selectivity of PICK1 antibody used in immunogold labeling is characterized previously (Haglerød et al., 2009). The anti-GluA2/3 antibody has been used in several recent immunocytochemical studies, which all have shown the expected characteristic staining pattern (Edwards et al., 2013; Sadeghi et al., 2014; Schuth et al., 2014; Peng et al., 2015).

Animals

Wistar male rats weighing 250–300 g (Møllegaard, Ejby, Denmark) were used for EM and LM. PVG male rats, 2–4 months, (Scanbur, Nittedal, Norway), weighing 200–250 g, were used for WB. The animals were allowed free access to food and water. Experimental protocols were approved by the Norwegian Institutional Animal Care and Use Committee and conform to National Institutes of Health guidelines for the care and use of animals. The organotypic cultures were prepared from p8 male Wistar rats, in accordance with British animal protection legislation and the British

National Committee for Ethics in Animal Research approved the experimental protocols.

Immunocytochemistry

Perfusion fixation: For electron microscopy, the rats (n=3) were deeply anaesthetized with Equithesin (0.4 ml/100 g body weight) followed by intracardiac perfusion with 10-15 seconds flush of 4% Dextran-T70 in sodium phosphate buffer (pH 7.4) followed by a mixture of 4% FA and 0.1% GA in the same buffer. Small (0.5 x 0.5 mm) blocks from CA1 were freeze substituted, sectioned, and immunolabeled as described previously (Mathiisen et al., 2006). For double labeling, the sections were first incubated with rabbit polyclonal anti-PICK1 followed by goat anti-rabbit coupled to 20 nm colloidal gold. The sections were exposed to formaldehyde vapor at 80 C for 1 h and thereafter incubated with rabbit polyclonal anti-GluA2, followed by goat anti-rabbit coupled to 10 nm colloidal gold. The sections were examined with a Fei Tecnai 12 electron microscope at 60 kV. For bright field light microscopy the rats were perfused as above, but glutaraldehyde was omitted from the fixative. Vibratome sections were cut from whole brain tissue.

Electron microscopy quantification and statistical analysis: Electron micrographs of asymmetric structurally defined synapses were obtained randomly from the middle layer of the stratum radiatum in the CA1 region of the hippocampus (Schaffer collateral synapses). Images of 60 synapses from each animal were selected for analysis; each showed intact plasma membranes, presynaptic vesicles and a clear synaptic cleft. GluA2 immunolabeling was quantified as number of gold particles/μm (linear density) of membrane length or as number of gold particles/μm^2 (area density) for regions of interests in the intracellular compartment in asymmetric synapses. Specific plasma membrane and cytoplasmic compartments were defined as regions of interests (ROIs) and used for quantification. The ROIs were: the postsynaptic density membrane (PSD), the active zone (AZ), the postsynaptic lateral membrane (PoL), i.e., the plasma membrane on each side of the PSD, the presynaptic lateral membrane (PreL), i.e., the plasma membrane on each side of the active zone, the postsynaptic cytoplasm (PoCy), the presynaptic cytoplasm (PreCy), and the astrocyte cytoplasm (ACy). For all synapses, the synaptic lateral membranes were defined for convenience of measurement as equal to the length of the PSD, on both sides of the PSD or active

zone. An in-house extension to analySIS software connected with SPSS (SPSS Inc, Chicago, Illinois, USA) was used to evaluate the gold particle labeling of the specific plasma membrane and cytoplasmic compartments. The software calculated linear particle density (number per unit length of curve) over membrane domains and area particle density (number per unit area) over cytoplasmic compartments. In the first case, it measured the distance from each particle center to the membrane and included only those particles that were within an operator-defined distance from the curve segment. For plasma membranes the inclusion distance was symmetric between -21 nm, and + 21 nm (negative signifying an intracellular location). In order to easily compare regions of interests, linear densities (particles/μm) were recalculated, using the inclusion distance from the middle of the plasma membrane, to area particle densities (particles/μm^2). Data for particles were collected in ASCII files as flat tables and exported to SPSS for further statistical and graphical analysis. Note that in juxtaposed membranes, i.e., the active zone and postsynaptic density membrane (PSD), the inclusion zones partly overlap. Thus, the calculated gold particle density values for each of these two ROIs are somewhat overestimated.

Organotypic slice cultures and viral infection

Organotypic hippocampal slices were cultured according to the standard interface method (Stoppini et al., 1991). Transverse 350 μm thick hippocampal slices were prepared on a McIlwain tissue chopper (The Mickle Laboratory Engineering, UK) from p8 male Wistar rats under semi-sterile conditions. Each slice was transferred onto Millicell-CM membrane inserts (Millipore, MA, USA, Cat#PICM03050) and the cultures were maintained at 37^o C humidified 95% air and 5% CO2. The culture media consisted of 75% minimal essential medium (MEM) (Thermo Fisher scientific, MA, USA) 25% heat inactivated horse serum (Gibco, Thermo Fisher) and glucose (6.5 g/l). The medium was changed twice a week. After 7 days, Sindbis virus containing inserts for green fluorescent protein (GFP), pep2m, or GFP-PICK1, was pressure injected using sterile Femtotips II microcapillaries (Eppendorf, Hamburg, Germany) mounted on an automated FemtoJet and InjectMan NI2 microinjection system (Eppendorf, Hamburg, Germany) into the CA1 pyramidal cell layer of each slice before being placed back into the incubator. The recombinant attenuated Sindbis viruses were produced as per manufacturers instructions (Invitrogen, Thermo Fisher). Briefly, RNA for Sindbis virus constructs (pSinRep5) encoding GFP, GFP-PICK1

and pep2m were prepared by in vitro translation and electroporated into BHK-21 (baby hamster kidney cells) with DH26S (defective helper). Cells were spun down and supernatant containing pseudovirion was kept at -20° C until use. Slices were fixed 24 h after infection with 4% formaldehyde (FA) and 0.1 % glutaraldehyde (GA) in sodium phosphate buffer (pH 7.4). Small (0.5 x 0.5 mm) blocks from CA1 were freeze substituted, sectioned, and immunolabeled as described previously (Mathiisen et al., 2006). The cultures used in the present study were taken from a pool of cultures transfected for further immunofluorescence investigations. These confirmed the high efficiency of the virus infection. For subsequent immunogold quantifications it was impossible to monitor the transfection of the individual neurons or synapses. Thus, the selected pool of synapses would originate from both transfected and not transfected neurons. The resulting differences in labeling levels between transfected and non-transfected groups will then most likely be an underestimate, since non-transfected synapses will be included in the transfected group as well. Three hippocampal slices from each group were used for analysis. Sixty profiles (20 profiles from each slice) from each region of interest were quantified. The ROIs used for quantification were: The postsynaptic density membrane (PSD), the active zone (AZ), the postsynaptic cytoplasm (PoCy) and the presynaptic cytoplasm (PreCy).

Bright field microscopic studies

Free floating vibratome sections from rat brain (50 µm) were treated with 1 M ethanolamine-HCL (pH 7.4), blocked with 3 % (v/v) normal calf serum (NCS) in 0.1 M sodium phosphate buffer pH 7.4, and incubated with primary antibodies, overnight at room temperature (ON/RT), followed by incubation with secondary antibodies for 1 hour at RT and development with the biotin-streptavidin-peroxidase system and 3,3–Diaminobenzidine (DAB).

Immunoblotting

Four rats were sacrificed and different brain regions were dissected out. The homogenates of different brain regions were run on 4-20 % SDS-acrylamide gels, then electroblotted onto PVDF membrane (Hoefer Scientific Instruments, San Francisco, CA, USA) and immunostained with primary and secondary antibodies. The signal was detected by fluorescence using ECF substrate (Amersham biosciences,

UK). Quantitative analysis of blots was performed using Photoshop (Adobe) and Excel (Microsoft). Staining of different brain regions with anti-PICK1 and anti-GluA2 were normalized against β-tubulin staining. Three blots for each protein were used for quantitation. A rectangular marker was made so that it could tightly fit the bands. Values for means and pixels were transferred to Excel sheet. The background was measured and subtracted from "mean".

Results

GluA2 and PICK1 are strongly, but differentially, expressed in synapse rich areas of the brain

We reasoned that brain areas with the highest density of AMPARs and PICK1 would be most amenable to subsequent EM analysis of the ultrastructural localization of these proteins. Therefore, to determine relative levels of GluA2 and PICK1 in the brain, we performed quantitative western blot analysis of different rat brain regions. There are distinct differences in total GluA2 expression between different brain regions (Fig. 1B,D). The hippocampus clearly contains the highest concentration of GluA2, at 40% stronger mean band intensity than the cerebral cortex. The thalamus, brainstem and cerebellum show intermediate levels of GluA2, at 53%, 41%, and 60% of the cortex, respectively. The spinal cord shows only low GluA2 concentrations, at only 12% of the cortex.

PICK1, however, is relatively uniformly expressed in the cortex, thalamus, and hippocampus, with the thalamus and hippocampus at 97% and 91% of the cerebral cortex, respectively (Fig. 1C,E). The brainstem and cerebellum showed intermediate levels of PICK1, at 74% and 86% of the cortex, respectively. The lowest concentration of the protein was found in the spinal cord, at 52% of the mean band intensity found in the cortex. Clearly, the variability of PICK1 expression among different brain regions is lower than that seen for GluA2.

We next investigated the cellular distribution patterns of GluA2 and PICK1 in different brain regions. Bright field light microscopy (Fig. 2) revealed strong immunoreactivity for both PICK1 and GluA2 in the cerebral cortex, hippocampus, and thalamus (Fig. 2A1,A2,B1,B2), corresponding to our WB data. At higher magnifications, somata and proximal dendrites of cortical and hippocampal pyramidal cells, thalamic neurons and cerebellar Purkinje cells were all strongly immunopositive for both proteins, as were granule cells of the dentate gyrus (Fig. 2A3-8; 2B3-8), confirming the neuronal origin of the WB and immunohistochemical signal.

GluA2 and PICK1 are associated with presynaptic vesicles and the plasma membrane of the active zone in glutamatergic synapses

Consistent with previous reports, our data confirm that the hippocampus strongly expresses both GluA2 and PICK1. Therefore, we used hippocampus for ultrastructural EM analyses of the presynaptic expression pattern of GluA2. We used postembedding immunogold labeling of thin sections of the middle levels of the stratum radiatum in the CA1 region of the hippocampus with anti-GluA2 antibody (Fig. 3A,C,D). Firstly, electron micrographs confirmed that GluA2 is present in postsynaptic spines, both in the postsynaptic density (PSD) region, and in the spine cytoplasm. Secondly, we observed the GluA2 subunit associated with the membrane of the active zone and in the presynaptic cytoplasm, associated with vesicles in asymmetric synapses. These were regarded as Schaffer-collateral synapses.

We also performed postembedding immunolabeling of ultrathin sections with anti-PICK1 antibody in the same area to determine the synaptic localization of this protein (Fig. 3B,C,D). Postsynaptically, PICK1 was associated with spine cytoplasm and the PSD. PICK1 was also present presynaptically, with gold particles associated with cytoplasm, vesicles and the active zone. Some gold particles were observed at perisynaptic lateral membranes, both pre and postsynaptically.

Double immunogold labeling with anti-GluA2 and anti-PICK1 antibodies were performed to determine if PICK1 and GluA2 were colocalized in the same subcellular regions. Electron micrographs displayed the presence of GluA2 and PICK1 together in the same glutamatergic synapses (Fig. 3C,D). Both proteins were localized in pre- and postsynaptic vesicles in the same synapses. Colocalization of the proteins was also seen at the active zone (AZ) and the PSD. These results demonstrate that AMPA receptor GluA2 subunits are expressed in the presynaptic terminal as well as in the postsynaptic spine and colocalized with PICK1.

Quantitative postembedding analysis of GluA2 expression in the synapse

We subsequently performed an analysis of the number of GluA2-associated gold particles as a function of the distance from a line drawn through the centre of the synaptic cleft, between the pre- and postsynaptic plasma membranes (transverse

histogram). As is evident form Fig. 4B there was a large peak of gold particles associated with GluA2 over the PSD and the adjacent spine cytoplasm, and a somewhat smaller peak of gold particles over the active zone, with a low-value cleft between them, showing that GluA2 is present in both the synaptic plasma membranes.

Next, we compared the GluA2 labeling intensities over different subsynaptic regions (see Fig. 4A for a schematic illustration of the different regions analyzed), through a quantitative distribution analysis of the gold particles. As is evident from Fig. 4C, the density of gold particles associated with GluA2 is high both in the PSD, where the mean number of gold particles per μm^2 was 73, and in the active zone, where the mean number of gold particles per μm^2 was 67. Thus, both the synaptic plasma membranes were clearly more strongly labeled for GluA2 than the plasma membranes lateral to the synaptic cleft: The lateral postsynaptic plasma membrane showed a mean number of gold particles per μm^2 of 31, and the lateral presynaptic plasma membrane showed a mean number of gold particles per μm^2 of 25.

In the cytoplasmic compartments, the level of GluA2 presynaptic labeling (15 gold particles/μm^2) is almost as high as in the corresponding postsynaptic cytoplasm (17 gold particles/μm^2), but both are clearly higher than in adjacent astrocytic cytoplasm (6 gold particles per μm^2) (Fig. 4D). Thus, in addition to its established presence in postsynaptic spines, GluA2 appears to be associated with plasma- and vesicle membranes in presynaptic compartments of asymmetric synapses.

Presynaptic trafficking of GluA2 is regulated by PICK1 and NSF

To determine if PICK1 and NSF are involved in the trafficking of the AMPA receptor subunit GluA2 in presynaptic terminals, as established for postsynaptic spines, we performed viral infections of organotypic hippocampal cultures with pep2m and GFP-PICK1, using separate infections with GFP as control. The peptide pep2m inhibits the interaction between GluA2 and NSF by binding to the NSF binding site on the C terminal of GluA2 (Nishimune et al., 1998; Noel et al., 1999; Collingridge and Isaac, 2003). We immunolabeled ultrathin sections from each infection group with antibodies against GluA2/3 (Fig. 5A,B,C) and performed quantitative immunogold analysis of the PSD, active zone, and post- and presynaptic cytoplasm in the stratum radiatum of the CA1 region. The analysis (Fig. 5D,E) showed that in both

overexpressing PICK1 or blocking NSF-GluA2 interactions with pep2m significantly reduce GluA2/3 immunogold labeling within the PSD, the active zone and the postsynaptic cytoplasm compared with the control group (GFP). In the presynaptic cytoplasm, the GluA2/3 labeling intensity was only significantly reduced in the PICK1 group, suggesting that NSF involved in partitioning of AMPARs to the active zone but not their general recruitment and localization within the presynaptic bouton. Increasing the concentrations of PICK1 by transfection serves to decrease synaptic expressions of GluA2. Transfection with pep2m, presumably inhibiting the NSF-GluA2 interaction, gives a similar result.

Discussion

Synaptic plasticity can be regulated at the presynaptic side by altering the efficacy of neurotransmitter release (Bliss and Collingridge, 1993; Bear and Malenka, 1994; Lisman, 2003; Enoki et al., 2009; Anggono and Huganir, 2012; Choquet and Triller, 2013). For a functional presynaptic regulation, one would expect both feedforward and feedback mechanisms in order to adjust the strength of neurotransmission. One way of achieving such a feedback function in synapses would be to insert AMPA receptors also in the presynaptic plasma membrane, acting as glutamate sensors.

The underlying question behind the present investigation is whether suitable synaptic feedback mechanisms are in place to sustain regulation of presynaptic release, in this case in the Schaffer collateral synapses in CA1 in the hippocampus. Specifically, this entails the presence of autoreceptors on the presynaptic plasma membrane, providing continuous information back to the terminal about the concentration of transmitter in the synaptic cleft. However, it will also necessitate molecular means of regulating the sensitivity of the presynaptic autoreceptor apparatus. An effective way of doing this is by increasing or decreasing the concentration of the autoreceptors in the presynaptic active zone.

We have investigated AMPAR localization within the presynaptic plasma membrane, by using antibodies against the AMPA receptor subunit GluA2. We found that GluA2 is present in the active zone, thereby providing a means of monitoring glutamate concentration in the synaptic cleft, and we found that presynaptic terminals also contain PICK1, which could provide a mechanism for regulating the concentration of GluA2 in the active zone. Different physiological functions of presynaptic glutamate receptors have been discussed previously (Pinheiro and Mulle, 2008). However, the presynaptic functions and mechanisms have been technically challenging to investigate. At the calyx of Held, in the rat auditory brainstem, activation of presynaptic AMPA receptors have been shown to induce inward currents in the nerve terminal and thus inhibit presynaptic Ca^{2+} currents, thereby attenuating glutamatergic synaptic transmission (Takago et al., 2005).

In the experiments used for quantification (Figs. 4, 5) we used an antibody that does not distinguish between GluA2 and GluA3. Thus, we cannot exclude that some of the quantified gold particles in fact represent GluA3 subunits. However, as referred to in the introduction, the vast majority of synaptic and extrasynaptic AMPA receptors contain GluA2, only a minority contains GluA3, and of these, most are GluA2-GluA3 heteromers. We also tested the presynaptic localization of GluA2 with a specific antibody (Fig. 3), clearly showing the presence of GluA2 in presynaptic terminals. Thus, the contribution of GluA3 subunits to our results should for all practical purposes be small.

Initially we used western blotting (Fig. 1) and light microscopical immunohisto/cytochemistry (Fig. 2) to confirm the presence of GluA2 and PICK1 in neurons of various brain regions. While these methods did not enable us to distinguish between pre- and postsynaptic receptors, they did show us that compared to various brain regions, the hippocampus is enriched in GluA2 and PICK1. Our regional GluA2 results were consistent with previous studies (Petralia and Wenthold, 1992; Martin et al., 1993) but, to our knowledge, this is the first work that compares PICK1 levels in different brain regions in a quantitative manner.

High concentrations of AMPA receptors may be expected in areas of the brain with high demands for synaptic plasticity, like the hippocampus. On the other hand, as is evident from the light microscopical immunocytochemical staining experiments (Fig. 2A), most of the expressed GluA2 protein is localized within the intracellular somatic/dendritic cytoplasm, and not in the synapses. The synaptic pool of GluA2, specifically, is more relevant for discussing the functional role of the receptor subunit than the overall hippocampal or brain concentration.

In the main part of our study, we then used quantitative, high-resolution immunogold electron microscopy to investigate subsynaptic concentrations of GluA2 and PICK1, and also the interplay between these two proteins in the pre- and postsynaptic compartments, in addition to the role of NSF in these regulatory processes. The immunogold labeling patterns (Fig. 3 and 4) confirm that GluA2 is present presynaptically as well as postsynaptically. There are minor differences between the densities of GluA2 gold particles in the PSD vs the AZ and the postsynaptic

cytoplasm vs the presynaptic cytoplasm. On the other hand, both the synaptic plasma membrane regions have remarkable higher densities of GluA2 gold particles than the parts of the membranes lateral to the PSD or AZ. These observations are entirely consistent with presynaptic AMPAR functions. Interestingly, although astrocytes have been shown to express functional AMPARs (Seifert and Steinhauser, 2001; Dzamba et al., 2015), we show here that the concentration of AMPA receptors in astrocytes is only about a third of that found in neuronal synapses.

We have previously reported the presence of GluA2 in presynaptic terminals in the CA1 region of the hippocampus (Feligioni et al., 2006; Haglerød et al., 2009). Several others have reported the presence of presynaptic AMPARs in other CNS synapses (Satake et al., 2000; Schenk et al., 2003; Takago et al., 2005; Chavez et al., 2006; Pittaluga et al., 2006; Summa et al., 2011; Grilli et al., 2012). However, this is the first quantitative analysis of the intrasynaptic distribution of this AMPAR subunit. Most importantly, we show that the concentrations of presynaptic pools of GluA2 are comparable to those of the postsynaptic spine, and that the active zone harbors significant concentrations of this AMPAR subunit. The presence of GluA2 in the presynaptic active zone and cytoplasm is compatible with the hypothesis that GluA2 functions as a presynaptic autoreceptor to modulate transmitter release. In an influential analysis of the protein content of synaptic vesicles (Takamori et al., 2006), neither GluA2, GluA3, or PICK1 are mentioned. However, this study used a synaptic vesicle preparation from whole rat brain, and the proteins were found by Coomassie blue staining after gel electrophoresis. If presynaptic AMPA receptors only occur in a subset of synapses, there would be little chance of identifying them with Coomassie blue from a whole brain.

We also confirm (Haglerød et al., 2009) that PICK1 co-localizes at the ultrastructural level with GluA2 (Fig. 3C,D). Both proteins are present on pre- and postsynaptic vesicles, in many cases the same vesicles. Furthermore, our data show that overexpression of PICK1 and pep2m significantly reduces the immunogold labeling intensity of GluA2 over synaptic membranes (Fig. 5). These data are consistent with the possibility that PICK1 and NSF interact with, and regulate the local trafficking of GluA2 in presynaptic terminals, as they have been shown to do postsynaptically (Anggono and Huganir, 2012; Henley and Wilkinson, 2013). More specifically, our

data are compatible with a model where PICK1 facilitates internalization of GluA2, from the active zone to the intracellular pool, while NSF facilitates insertion of GluA2 from the intracellular pool to the active zone. We have previously shown GluA2- and PICK1-staining of synaptic vesicle fractions (Haglerød et al., 2009), but we still cannot exclude that some of the immunogold labeling seen in the present investigation could represent endosomes or other presynaptic organelles.

The expression of GluA2 within the active zone ensures proximity to sites of vesicular release. Upon release of transmitter from a single vesicle, the concentration of glutamate within the synaptic cleft may by some estimates, about 1 mM (Clements et al., 1992), be sufficiently high to activate all the AMPA receptors within the membrane of the presynaptic active zone. One study has in fact provided support for presynaptic AMPA receptors providing a positive feedback system at presynaptic, glutamatergic synapses that could play important roles in plasticity as well as in excitotoxic processes (Patel and Croucher, 1997).

At the postsynaptic site, both PICK1 and NSF contribute to regulating GluA2 insertion and retraction from the plasma membrane in a calcium dependent manner (Hanley and Henley, 2005). Our data suggest that PICK1 and NSF may play a similar role in the presynaptic terminal, where overexpression of PICK1, as well as expression of pep2m, reduces the labeling intensity of GluA2 at plasma membrane and cytoplasmic sites. The reduction in labeling intensity over cytoplasmic areas may reflect retrograde transport of the receptors from the terminal after retraction from the plasma membrane.

The absolute number of synaptic gold particles obtained with GluA2/3 antibody was higher in the organotypical cultures than in intact brain tissue (Fig. 5 vs Fig. 4). The difference was more strongly pronounced in the plasma membrane compartments (PSD and AZ) than in the cytoplasmic compartments. The main reason for the difference is probably that these tissues were treated very differently with regard to fixation procedures, but also that the intact brain sections and the organotypical sections were labeled in different experiments. However, due to the different ages of the animals, as well as the difference between intact brain and cultures, there may be a real difference in the intrasynaptic distribution between plasma membrane and

cytoplasmic AMPA receptor pools.

The technical challenges with transfecting the pre- and postsynaptic elements in separate experiments were greater than we were able to successfully attack in the present investigation. The Schaffer collateral axons pass through the CA1 area to terminate on the pyramidal cell apical dendrites. The consequence is that virus that is injected into the stratum pyramidale can also transfect axons passing through this area. The distance from the injection site to the synapses we have investigated is only about 100 μm. Axons and terminals harbor the molecular machinery necessary for local protein synthesis (Crispino et al., 2014), so that by virus injection at a single site, the proteins were expressed in both the pre- and postsynaptic elements.

According to our hypothesis that presynaptic AMPA receptors have a role as autoreceptors on the extracellular side of the synaptic plasma membrane, the ligand-bidning site will then be localized on the luminal side of the corresponding synaptic vesicles. These vesicles contain high concentrations of glutamate. However, due to the rapid desensitization and closing of AMPA receptors after binding of glutamate (Jones and Westbrook, 1996), we would expect little leakage of ions from the vesicles through the receptor channels.

One obvious intriguing question is whether the GluA2/PICK1-associated presynaptic vesicles represent a distinct sub-pool of vesicles in the terminal, or whether they are identical to the glutamate containing vesicles that are used for neurotransmitter release. We do not have any data that may answer this question. The possibility exists then, that upon vesicular glutamate release, glutamate receptors are simultaneously and automatically inserted into the presynaptic plasma membrane along the active zone. On the other hand, if GluA2 is present on a subset of presynaptic vesicles, there might exist distinct exocytotic regulatory mechanisms for these vesicles, independent of transmitter release itself. Further studies are needed to determine between these alternatives.

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Figure legends

Fig. 1. Western blots of homogenates from different brain regions, i.e., cortex [Cx], thalamus [Th], hippocampus [Hc], brain stem [Bs], cerebellum [Cb], and spinal cord [Spc]. (A) Staining of different brain regions were normalized against β-tubulin staining. (B) Staining with anti-GluA2. (C) Staining with anti-PICK1. (D) Quantitation of GluA2 labeling intensities. (E) Quantitation of PICK-1 labeling intensities. Error bars denote SEM, (n = 3).

Fig. 2. Light micrographs of rat brain vibratome sections, stained with immunoperoxidase method, with anti-GluA2 (A) and anti-PICK1 (B). The upper left image in each of the panels is coronal sections showing cortex, hippocampus, and thalamus. Hc: hippocampus. CA1, CA3 and DG (dentate gyrus) are subregions of the hippocampus. Cx: cerebral cortex. Th: thalamus. Cb: cerebellum. Note neurons with dense staining throughout the cytoplasm and large dendrites. Scale bars: Brain 2 mm. Hc 500 μm. CA1, CA3, DG, Th 20 μm. Cx 50 μm. Cb 25 μm.

Fig. 3. Electron micrographs of asymmetric synapses in the stratum radiatum in CA1 of the hippocampus. Immunogold labeling with anti-GluA2 (A) and anti-PICK1 (B). (C,D) Double immunogold labeling for PICK1 (20 nm) and GluA2 (10 nm). Note labeling of pre- and postsynaptic vesicles. Abbreviations: AT, axon terminal; PS, postsynaptic; AZ, active zone; PSD, postsynaptic density. Scale bars: A, B, C: 125 nm. D: 50 nm.

Fig. 4. Quantification of immunogold localization after GluA2/3 labeling in different subcellular compartments of asymmetric stratum radiatum synapses in CA1 of the hippocampus. (A) Schematic illustration of a synapse, with seven specific membrane or cytoplasmic compartments that were subjected to quantitative analysis of gold particle labeling: postsynaptic density membrane (PSD), active zone (AZ), postsynaptic lateral membrane (PoL), presynaptic lateral membrane (PreL), postsynaptic cytoplasm (PoCy), presynaptic cytoplasm (PreCy), and astrocyte cytoplasm (ACy). The length of the lateral membranes included for quantification

correspond to half of the total length of the PSD or the active zone on each side. (B) Transverse histogram showing the number of gold particles at different distances (nm) from the middle of the synaptic cleft (zero), in postsynaptic (negative values), and presynaptic (positive values) directions, respectively. The histogram shows that the immunogold particles are strongly concentrated over the postsynaptic density, while a slightly lower immunogold labeling is seen over the active zone. (C) Quantitative assessment of labeling density over four different plasma membrane compartments indicated in A. (D) Quantitative assessment of labeling area density in three different cytoplasmic membrane compartments indicated in A. Values along the abscissa gives the density of gold particles/μm^2 ± 95% confidence interval in C and D (n = 60).

Fig. 5. Quantitative immunogold analysis of GluA2/3 labeling intensity of asymmetric synapses in stratum radiatum of organotypic cultures of the hippocampus (CA1 region) virally infected with PICK1, pep2m and GFP (control). (A-C) Electron micrographs showing immunogold labeling of synapses infected with (A) GFP, (B) pep2m, (C) PICK1. (D) Analysis of gold area density (number of gold particles/μm2) was performed over the postsynaptic density (PSD) and the active zone (AZ). (E) Analysis of gold particle area density (number of gold particles/μm2) was performed over the postsynaptic cytoplasm (PoCy), and the presynaptic cytoplasm (PreCy). Note that the labeling intensity was significantly reduced in both transfection groups, i.e. PICK1 (white bars) and pep2m (light grey bars), compared to the control (dark grey bars) over the postsynaptic density, the presynaptic active zone and the postsynaptic cytoplasm. In the presynaptic cytoplasm the labeling intensity was only significantly reduced in the PICK1 group. Statistical significance ($p<0.05$) indicated with an asterisk. Error bars show 95% confidence interval (CI) (n = 60). Scale bars: 200 nm.

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