Rubio, M., Matsui, K., Fukazawa, Y., Kamasawa, N., Harada, H., Itakura, M., Molnar, E., Abe, M., Sakimura, K., & Shigemoto, R. (2017). The number and distribution of AMPA receptor channels containing fast kinetic GluA3 and GluA4 subunits at auditory nerve synapses depend on the target cells. *Brain Structure and Function, 222*(8), 3375–3393. https://doi.org/10.1007/s00429-017-1408-0

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The number and distribution of AMPA receptor channels containing fast kinetic GluA3 and GluA4 subunits at auditory nerve synapses depend on the target cells

María E. Rubio1,2,3 · Ko Matsui4 · Yugo Fukazawa5 · Naomi Kamasawa6 · Harumi Harada7 · Makoto Itakura8 · Elek Molnár9 · Manabu Abe10 · Kenji Sakimura10 · Ryuichi Shigemoto7

Received: 17 August 2016 / Accepted: 20 March 2017 © The Author(s) 2017. This article is an open access publication

Abstract The neurotransmitter receptor subtype, number, density, and distribution relative to the location of transmitter release sites are key determinants of signal transmission. AMPA-type ionotropic glutamate receptors (AMPARs) containing GluA3 and GluA4 subunits are prominently expressed in subsets of neurons capable of firing action potentials at high frequencies, such as auditory relay neurons. The auditory nerve (AN) forms glutamatergic synapses on two types of relay neurons, bushy cells (BCs) and fusiform cells (FCs) of the cochlear nucleus. AN-BC and AN-FC synapses have distinct kinetics; thus, we investigated whether the number, density, and localization of GluA3 and GluA4 subunits in these synapses are differentially organized using quantitative freeze-fracture replica immunogold labeling. We identify a positive correlation between the number of AMPARs and the size of AN-BC and AN-FC synapses. Both types of AN synapses have similar numbers of AMPARs; however, the AN-BC have a higher density of AMPARs than AN-FC synapses, because the AN-BC synapses are smaller. A higher number and density of GluA3 subunits are observed at AN-BC synapses, whereas a higher number and density of GluA4 subunits are observed at AN-FC synapses. The intrasynaptic distribution of immunogold labeling revealed that AMPAR subunits, particularly GluA3, are concentrated at the center of the AN-BC synapses. The central distribution of AMPARs is absent in GluA3-knockout mice, and gold particles are evenly distributed along the postsynaptic density. GluA4 gold labeling was homogenously distributed along both synapse types. Thus, GluA3 and GluA4 subunits are distributed at AN synapses in a target-cell-dependent manner.

Keywords Electron microscopy · Ventral cochlear nucleus · Synapses · Bushy cells · Fusiform cells · Postsynaptic density · Freeze-fracture replica immunolabeling

Electronic supplementary material The online version of this article (doi:10.1007/s00429-017-1408-0) contains supplementary material, which is available to authorized users.

© Maria E. Rubio mer@pitt.edu

1 Department of Otolaryngology, University of Pittsburgh Medical School, BST3 Building, 3501 Fifth Avenue #10016, Pittsburgh, PA 15261, USA
2 Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, USA
3 Center for the Neural Basis of Cognition, University of Pittsburgh, Pittsburgh, PA, USA
4 Division of Interdisciplinary Medical Science, Tohoku University, Sendai, Japan
5 Department of Brain Structure and Function, Faculty of Medical Sciences, University of Fukui, Fukui, Japan
6 Max Planck Florida Institute, Jupiter, FL, USA
7 IST Austria, 3400 Klosterneuburg, Austria
8 Department of Biochemistry, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan
9 School of Physiology, Pharmacology and Neuroscience, University of Bristol, Biomedical Sciences Building, Bristol BS8 1TD, UK
10 Niigata University Brain Research Institute, Niigata, Japan

Published online: 10 April 2017
Introduction

Diverse information is embedded within the spike trains of each neuron, and the properties of the signals transmitted at individual synapses are at least partially tuned, such that the information encoded in the spike trains can be appropriately transmitted to and interpreted by the postsynaptic target neurons. Neurotransmitters released from presynaptic neurons diffuse to activate their receptors expressed on postsynaptic cell membranes. A range of receptor subtypes has been identified for each neurotransmitter, and these subtypes differ in their molecular organization and pharmacological and biophysical properties, such as their affinity for the transmitter, associated signaling mechanisms, and temporal kinetics of receptor activation and inactivation. Therefore, the type, number, density, and distribution of receptors in a synapse likely shape responses at individual synapses. Here, we identify the differential regulation of the expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtypes in two different types of postsynaptic neurons that are activated by the same type of presynaptic neurons. We propose that the distinct receptor organization patterns observed in these synapses may underlie the differential retrieval of distinct information from the spike trains, which, in turn, results in the processing of distinct information by these target neurons.

Ionotropic AMPA-type glutamate receptors (AMPARs) mediate fast excitatory transmission along the ascending auditory pathway (Raman et al. 1994). AMPARs are tetrameric complexes composed of homologous or heterologous combinations of GluA1–4 subunits. The electrophysiological properties of the AMPAR channel vary substantially depending on the subunit compositions (Holmman and Heinemann 1994). For example, GluA1-dominant AMPARs have slow gating characteristics, and these receptors have critical roles in neuronal growth, long-term potentiation, and cognitive functions (Derkach et al. 2007). GluA2-lacking AMPARs are highly permeable to Ca$^{2+}$ and have submillisecond gating kinetics. These receptors are prominently expressed in subsets of neurons that are capable of firing action potentials at high frequencies, such as auditory relay neurons (Geiger et al. 1995; Raman et al. 1994).

The mature auditory nerve (AN) forms synapses with both bushy cells (BCs) of the ventral cochlear nucleus and fusiform cells (FCs) of the dorsal cochlear nucleus. Both synapses contain GluA3 and GluA4 subunits, allowing AMPARs to rapidly respond to released glutamate (Rubio and Wenthold 1997, 1999; Wang et al. 1998; Gardner et al. 1999, 2001; Rubio 2006; Whiting et al. 2009). Compared with AN-FC synapses, AN-BC synapses require extremely rapid synaptic transmission to preserve information contained in the timing of the AN spikes (Gardner et al. 1999; Fujino and Oertel 2003). Thus, the GluA3/GluA4 ratio and the absolute number of these subunits may be specifically tuned at each synapse to meet the demands of the information they must transmit.

The alignment of receptors with presynaptic release sites may influence the probability and timing of receptor activation (Franks et al. 2003; Lisman et al. 2007; Tang et al. 2016). Vesicular release of glutamate in close proximity to high-density AMPAR subdomains (ON cluster release) is likely to elicit larger synaptic responses than glutamate release at low-density AMPAR subdomains (OFF cluster release). Simulations of the dorsal lateral geniculate nucleus (dLGN) have indicated that ON cluster release tends to cause a larger response than OFF cluster release (Tarusawa et al. 2009). Release at the center and periphery of the postsynaptic specialization also appears to produce different response amplitudes. Based on the observations obtained from these simulations, the primary determining factor that governs the amplitude of the synaptic response is the total number of AMPARs expressed, followed by their density. The difference in the response produced by the precise distribution of AMPARs within the postsynaptic specialization appears to be small (Tarusawa et al. 2009). However, the responses produced in the center or periphery may differ, depending on the receptor subunits, which have different response kinetics and dose–response curves. Moreover, the receptor distribution may have a greater influence if release always occurs in close proximity to the AMPAR cluster area or the center or periphery of the synaptic specializations.

Freeze-fracture replica immunogold labeling (FRIL) has been used to determine the localization of receptors within the postsynaptic density (PSD) with extremely high precision. The tangential distribution of synaptic AMPAR subunits has been examined in several synapses in the central nervous system (CNS) using postembedding immunogold methods (Matsubara et al. 1996; Bernard et al. 1997; Jacob and Weinberg 2015). However, the two-dimensional intrasynaptic distribution of AMPAR subunits has only been investigated with FRIL in a few synapses, including dLGN synapses and calyx of Held synapses (Budisantoso et al. 2012, 2013). In the dLGN, two presynaptic terminals from retinogeniculate synapses and corticogeniculate synapses that target onto dLGN relay cells were examined and compared (Tarusawa et al. 2009). Here, we examined AN synapses on BCs and FCs. The same presynaptic spike train likely propagates to both synapses. Thus, the expression of postsynaptic AMPAR subunits and their distribution differ in the two synapses to enable the extraction of specific aspects of information transmitted by the presynaptic AN. The importance of the synaptic architecture may be
established by examining the organization of these ultrafast auditory synapses.

**Materials and methods**

**Animals used for the morphological analysis**

For this study, male CD57B6J wild-type (WT) mice \((n=21)\) and newly developed GluA3-knockout (KO) \((n=3)\) and GluA4-KO \((n=3)\) mice were used at postnatal day 30. The mice were maintained on a 12 h light/dark cycle with water and food available ad libitum. All animal experiments were conducted in accordance with the guidelines of the University of Pittsburgh and Niigata University Animal Care and Use Committees.

**Generation of GluA3 and GluA4 KO mice**

Mice deficient in GluA3 or GluA4 were produced by homologous recombination using C57BL/6 embryonic stem (ES) cells (Supplemental Fig. 1). We isolated GluA3 \((\text{Gria3})\) and GluA4 \((\text{Gria4})\) genes from the C57BI/6 mouse genome using genomic PCR. A GluA3 targeting vector contained exon 11 of the Gria3 gene along with 4.2 kb upstream and 7.0 kb downstream homologous genomic DNA fragments and the diphtheria toxin gene for negative selection. A DNA fragment that carried a loxP sequence and pgk-1 promoter-driven neomycin phosphotransferase gene (Neo cassette) flanked by two Fp recognition target (ftr) sites was inserted into the site 107 bp upstream of exon 11. The pgk-1 polyadenylation (poly-A) signal sequence was inserted downstream of the Neo cassette. The other loxP site was introduced into a site 113 bp downstream of exon 11 to eliminate the putative transmembrane domain after Cre-mediated recombination. Homologous recombinant ES clones \((\text{Gria3}^{\text{flox/+}})\) were identified by Southern blot analysis. The SpeI-digested DNA hybridized with the 5’ probe and yielded a 19.7 kb product for the WT allele and a 9.8 kb product for the targeted allele; the DNA also hybridized with the neo probe to yield a 12.6 kb product for the targeted allele and the 3’ probe to yield a 19.7 kb product for the WT allele and a 12.6 kb product for the targeted allele.

The culture of ES cells and generation of chimeric mice were performed as previously described (Mishina and Sakimura 2007). Briefly, to establish the homologous recombinants, we introduced the linearized targeting vector into the C57BL/6-derived ES lines and subsequently selected recombinant clones with medium that contained 175 μg/mL G418. The targeted clones were microinjected into eight cell-stage embryos of the CD-1 mouse strain. The resulting chimeric embryos were developed to the blastocyst stage by incubating them for more than 24 h and were subsequently transferred to a pseudopregnant CD-1 mouse uterus. Germline chimeras were crossed with C57BL/6 female mice and the heterozygous offspring was crossed with TLCN-Cre mice (Nakamura et al. 2001; Fuse et al. 2004) to establish the GluA3 and GluA4 KO mouse lines.

All animal experiments were conducted in accordance with the guidelines established by the animal welfare committees and the ethics committees of Niigata University.

**FRIL**

Mice were anesthetized with ketamine and xylazine and transcardially perfused with 25 mM phosphate-buffered saline (PBS) for 1 min, followed by perfusion with 2% paraformaldehyde (PFA) and a 15% saturated picric acid solution in 0.1 M phosphate buffer (PB) for 12 min. Brains were immediately removed and placed in cold PBS. Coronal slices (130 μm thick) were cut using a vibrating microslicer (DTK-1000; Dosaka EM) in 25 mM PBS. The rostral anteroventral and dorsal cochlear nuclei (AVCN and DCN, respectively) were trimmed from the slice. The trimmed slices were immersed in 30% glycerol/25 mM PBS, incubated overnight at 4°C and rapidly frozen using a high pressure freezing machine (HPM010; BAL-TEC, Balzers; currently manufactured by RMC Boeckeler Instruments, Tucson, AZ). The frozen samples were then fractured into two parts at −140°C and replicated by the deposition of carbon (5 nm thick), platinum (uni-direction from 60°, 2 nm), and carbon (20 nm) in a freeze-fracture replica machine (BAF 060; BAL-TEC or JEOL JFDII, or JFDV). After thawing, the tissue debris attached to the replicas...
was dissolved in a solution containing 15 mM Tris–HCl (pH 8.3), 20% sucrose, and 2.5% SDS with gentle rocking for 18 h at 80°C. The replicas were subsequently washed with 50 mM Tris-buffered saline (TBS) (pH 7.4) containing 0.05% bovine serum albumin (BSA) and blocked with 5% BSA in washing buffer for 1 h at room temperature (~20°C). The replicas were incubated with rabbit primary antibodies against GluA1–4 (pan-AMPAR; Nusser et al. 1998), GluA3 or GluA4 (please refer to the “Antibody characterization” section) for 48 h at 15°C, followed by an overnight incubation with an anti-rabbit [British Biocell International (BBI)] secondary antibody conjugated with 5 nm gold particles at 15°C. The reliability of the AMPAR localization by FRIL under our fixation conditions has been discussed previously (Tarusawa et al. 2009).

**Antibody characterization**

Please refer to Table 1 for a list of all primary antibodies used in the present study. Rabbit polyclonal antibodies against GluA1–4 (pan-AMPAR), GluA3 and GluA4 were used.

The rabbit anti-AMPAR antibody (anti-GluA1–4 or anti-pan-AMPAR) was raised against a glutathione S-transferase (GST) fusion protein that contained the 58 extracellular amino-acid residues (724–781, Table 1) that preceded the last membrane-spanning segment of GluR1 flip (GST-GluA1 flip (724–781)). The preparation, purification, and full characterization of this antibody are described in the previous publications (Nusser et al. 1998; Pickard et al. 2000). The antisera were pre-adsorbed with the un-fused GST protein and subsequently affinity purified with the GST-GluA1 flip (724–781) fusion protein (Pickard et al. 2000). The affinity-purified rabbit polyclonal antibody detected the GST-GluA1 flip (724–781) fusion protein on immunoblots, and no cross-reactivity to GST was identified (Pickard et al. 2000). Furthermore, both antibodies immunoprecipitated the same 110 kDa proteins from solubilized rat brain membrane fractions, which were identified on immunoblots as AMPAR subunits using a panel of antibodies selective for the GluA1–4, GluA1, GluA2, and GluA3 proteins (Moul et al. 2006; Gladding et al. 2009). The FRIL patterns obtained with the rabbit anti-GluA1–4 antibody were entirely consistent with our previous reports (Tanaka et al. 2005; Masugi-Tokita et al. 2007; Antal et al. 2008; Tarusawa et al. 2009; Wang et al. 2014; Rubio et al. 2014). Using FRIL, parallel fiber-Purkinje cell synapses of GluA2/3 null mice were not labeled (Masugi-Tokita et al. 2007). Selective immunolabeling have repeatedly been observed in the postsynaptic membrane specialization of various synaptic connections in rat spinal cord (Antal et al. 2008), rat lateral geniculate nucleus (Tarusawa et al. 2009), mouse amygdala (Dong et al. 2010), and rat cochlear nucleus (Rubio et al. 2014). Based on these compelling observations, this antibody specifically labels all four subunits of AMPARs.

The rabbit polyclonal antibodies against GluA3 and GluA4 were raised using keyhole limpet hemocyanin-conjugated synthetic peptides. The following peptides were used: (C)NEYERFVPFSDDQQIS is located at the...
Intrasynaptic distribution of gold particles within the IMP cluster

The distributions of the GluA1–4, GluA3, and GluA4 immunoparticles within the demarcated IMP cluster were initially evaluated by creating a distance map from the border of the demarcation using the FIJI software [distributed under the General Public License (GPL)], as previously described (Budisantoso et al. 2012, 2013). Using this distance map, the IMP cluster area was divided into five divisions by placing contour lines at equal intervals (Figs. 7, 8, 9). An additional division outside of the demarcation (outer rim) with a 30 nm width was also created based on the potential spatial deviation of the immunoparticles from the antigen. The location of each immunoparticle was extracted from this distance map, and the density of immunoparticles in each division was tabulated (Figs. 7, 8, 9).

Identification of auditory nerve (AN) synapses on the replica

The AN synapses on the replicas of the AVCN and DCN were identified using a previously described method (Rubio et al. 2014). Errors in the identification of AN-BC and AN-FC synapses would imply that the true underlying distributions are even more different than the observed distributions.

Identification of AN-BC synapses on replicas of the AVCN

Only the most rostral sections of the anteroventral cochlear nucleus (AVCN) were used, because this area is enriched with BCs. The auditory nerve forms the main glutamatergic synapse on the cell bodies and dendrites of BCs (Gómez-Nieto and Rubio 2009, 2011; Sento and Ryugo 1989; Ryugo and Sento 1991). Membranes of BC dendrites were rarely observed in the AVCN replicas. In this study, we analyzed the IMP clusters of the AN synapses on the E-face membranes of BC somata (Fig. 1b). The IMP clusters on the E-face membrane of the BC somata were identified as previously described for a rat AVCN replica (Gulley et al. 1977; Rubio et al. 2014).

Identification of AN-FC synapses on replicas of the DCN

The DCN is a layered nucleus that is divided into a molecular or superficial layer (ML or layer I), a fusiform cell layer (FCL or layer II), and a deep layer (DL or layers III-IV). The procedure used to identify FCs and their basal dendrites was similar to that used previously (Rubio and Wenthold 1997; Rubio and Juiz 2004). The cell bodies of the
FCs are located in the FCL of the DCN and extend their apical and basal dendritic arbors towards the ML and DL, respectively.

**AN-FC synapses**

The AN fibers are the primary glutamatergic input within the FCL and DL that contact the FCs (Kane 1974; Smith and Rhode 1985; Ryugo and May 1993; Rubio and Wenthold 1997; Rubio and Juiz 2004). AN inputs form multiple synaptic contacts on the basal pole of the cell body and basal dendrites of FCs (Smith and Rhode 1985; Zhang and Oertel 1994; Rubio and Wenthold 1997). The IMP clusters located on the basal pole of the cell body of identified FCs and the proximal basal dendrites that were identified as extending from the cell body were analyzed (Fig. 2). The
IMP clusters on the E-face membrane of the FC basal dendrite were identified as previously described for a rat DCN replica (Rubio et al. 2014).

Measurement of the width of the postsynaptic membrane specialization from ultrathin sections

WT mice were anesthetized with a ketamine/xylazine mixture and transcardially perfused with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) with 2 mM calcium chloride at room temperature (~20°C). The brains were then removed and post-fixed in the same fixative solution for 2 h at 4°C. Coronal brainstem slices (80 μm thick) were vibratome-sectioned in an ice-cold solution (0.15 M cacodylate buffer and 2 mM calcium chloride). The sections were processed for electron microscopy using a previously described procedure (Rubio et al. 2014).

AN-BC and AN-FC synapses were identified based on their morphological features, as previously described (Rubio and Wenthold 1997; Rubio and Juiz 2004; Gómez-Nieto and Rubio 2009; Rubio et al. 2014). Serial images of the identified synapses were captured from the beginning to the end of each synapse at a magnification of 30,000× using a digital camera. The edge of the PSD was defined as a thickening of the postsynaptic membrane or the presence of IMP-clusters.
of a visible synaptic cleft, in addition to the rigid alignment of the presynaptic and postsynaptic membranes. The width and length of the PSD in each section were measured using ImageJ software (http://imagej.nih.gov/ij/). The maximum PSD width in each synapse was used for the analysis.

Data analysis

All measurements are reported as the means ± standard errors of the means (SEM) unless indicated otherwise. Statistical analyses were conducted using Prism 6 software (GraphPad Software, Inc.), and differences were considered statistically significant at p < 0.05. The normality of the data was assessed using Shapiro–Wilk’s W test. The statistical evaluation of the immunogold densities was performed using the Mann–Whitney U test or Kruskal–Wallis test when appropriate. The statistical evaluation of the maximum PSD and IMP cluster lengths was performed using the Mann–Whitney U test. Correlations were assessed using Pearson’s correlation test or Spearman’s rank-order test. One-way ANOVA was used to assess the differences in the intrasynaptic distribution of immunogold particles in each synapse type, and a simple two-way ANOVA test was employed to compare the intrasynaptic distribution between the WT and GluA3 KO mice.

Table 2 IMP-cluster areas analyzed for AN-BC in the AVCN and AN-FC in DCN

|          | AVCN  | DCN  |
|----------|-------|------|
| AN-BC    |       |      |
| Mean     | 0.031**** | 0.051 |
| SEM      | 0.001 | 0.003 |
| Median   | 0.028 | 0.042 |
| Kurtosis | 0.60  | −0.01 |
| Skewness | 0.89  | 0.87  |
| Minimum  | 0.005 | 0.008 |
| Maximum  | 0.078 | 0.123 |
| CV       | 0.50  | 0.56  |
| Count    | 94    | 83    |

****Statistically different between AN-BC vs AN-FC synapses. Mann–Whitney test p < 0.0001. Count refers to the total number of IMP-clusters analyzed.

Fig. 3 Differential distribution of AMPAR subunits in IMP clusters of AN synapses. FRIL images of IMP-clusters for auditory nerve-bushy cell (AN-BC) and auditory nerve-fusiform cell (AN-FC) synapses that were gold labeled (5 nm gold particles) for pan-AMPAR (GluA1–4), GluA3, or GluA4. The cartoons on the right show the distribution of the gold particles for two IMP clusters as representatives. The original size (5 nm) of the gold particles has been enlarged to aid visualization. Scale bar 200 nm

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in each synapse type, and a simple two-way ANOVA test was employed to compare the intrasynaptic distribution between the WT and GluA3 KO mice.
Results

Identification of AN synapses on bushy and fusiform cells

The postsynaptic membrane specialization of glutamatergic synapses in a FRIL image is indicated by a cluster of IMPs on the E-face of the plasma membrane (Sandri et al. 1972; Gulley et al. 1977; Harris and Landis 1986) and is often accompanied by the P-face of its presynaptic plasma membrane (Tarusawa et al. 2009; Rubio et al. 2014). The replicas were immunolabeled with specific antibodies against a conserved extracellular region for all AMPAR subunits (GluA1–4) or a peptide sequence unique to GluA3 or GluA4 (Table 1). In the rostral AVCN replicas, the IMP clusters were observed in the putative BC soma and were always immunopositive for the pan-AMPAR, GluA3, and GluA4 antibodies, confirming that these IMP clusters represent the postsynaptic specialization of glutamatergic synapses (Figs. 1, 2, 3). As reported for rats (Rubio et al. 2014), AN-BC and AN-FC synapses were often observed as multiple IMP clusters on large postsynaptic E-face membranes (Figs. 1, 2).
We investigated the synaptic morphology of glutamatergic postsynaptic membrane specializations using FRIL replicas of the rostral AVCN and DCN. We measured the areas of the IMP clusters that were entirely replicated on the fracture plane to compare the sizes of the synapses formed by the ANs on BCs and FCs. The putative PSDs of AN synapses exhibited qualitatively different morphologies in the arrangement of IMPs (Figs. 1, 2, 3). Quantitatively, the IMP cluster area of the AN-BC synapses was much smaller than that of the AN-FC synapses (Table 2, \( p < 0.0001 \), Mann–Whitney U test). Furthermore, we identified round (0.9 circularity; 0.7 roundness) IMP clusters with densely packed IMPs in the AN-BC synapses from the rostral AVCN. Within the DCN, more elliptical (0.6 circularity; 0.6 roundness) IMP clusters with a substantially sparser IMP distribution were frequently identified in the AN-FC synapses (Fig. 3).

We also analyzed the maximum widths of the PSDs from the AN-BC and AN-FC synapses in serial ultrathin

| Table 3 | Total number of gold particles for GluA1–4 (pan-AMPAR), GluA3, and GluA4 at AN synapses, together with the total number of IMP-clusters |
|--------|----------------------------------|
|        | AVCN | DCN |
|        | AN-BC | AN-FC |
| GluA1–4 |        |      |
| Total gold | 1232 | 1004 |
| IMP cluster N (positive/total) | 34/34 | 32/32 |
| GluA3 |        |      |
| Total gold | 734 | 231 |
| IMP clusters N (positive/total) | 30/30 | 23/23 |
| GluA4 |        |      |
| Total gold | 305 | 635 |
| IMP clusters N (positive/N) | 30/30 | 28/28 |

| Table 4 | Number and density of gold particles for GluA1–4, GluA3, and GluA4 at AN-BC and AN-FC synapses |
|--------|----------------------------------|
| GluA1–4 | AN-BC synapses | AN-FC synapses |
| IMP-clusters (n) |        |      |
| Number of gold particles/IMP-cluster | 36 ± 3 | 32 ± 3 |
| Mean (±SEM) | 35 | 26 |
| Median | 7–74 | 5–67 |
| Range |        |      |
| Density of gold particles/IMP-cluster (\( \mu m^2 \)) | 1100 ± 35 | 623 ± 47 |
| Mean (±SEM) | 1034 | 623 |
| Median | 732–1571 | 223–1257 |
| Range |        |      |
| GluA3 | IMP-clusters (n) | 30 | 23 |
| Number of gold particles/IMP-cluster | 25 ± 2 | 10 ± 1 |
| Mean (±SEM) | 21 | 9 |
| Median | 9–57 | 3–20 |
| Range |        |      |
| Density of gold particles/IMP-cluster (\( \mu m^2 \)) | 946 ± 50 | 184 ± 19 |
| Mean (±SEM) | 954 | 179 |
| Median | 500–1556 | 36–381 |
| Range |        |      |
| GluA4 | IMP-clusters (n) | 28 | 28 |
| Number of gold particles/IMP-cluster | 10 ± 1.2 | 23 ± 3 |
| Mean (±SEM) | 9.5 | 19 |
| Median | 1–22 | 5–44 |
| Range |        |      |
| Density of gold particles/IMP-cluster (\( \mu m^2 \)) | 337 ± 34 | 561 ± 38 |
| Mean (±SEM) | 372 | 539 |
| Median | 47–727 | 108–1091 |
| Range |        |      |
sections to verify that the areas of the IMP clusters in the replicas was comparable to the areas of the PSDs visualized in the conventional ultrathin sections (Fig. 4). The maximum width of the PSDs from the AN-BC synapses on the cell body (median 0.26 μm, n = 23 synapses) and that of the AN-FC synapses on proximal dendrites (median 0.32 μm, n = 27 synapses) were not significantly different from the maximum width of the IMP clusters in each of the two types of synapses (Fig. 4); (maximum width of the IMP clusters for the AN-BC synapse, median 0.25 μm, n = 38 synapses, and p = 0.5; for AN-FC synapses, median 0.31 μm, n = 31 synapses, and p = 0.51; Mann–Whitney U test). Based on the results from two different analyses, the IMP clusters on the E-face correspond to PSDs, and the average synapse size of the two excitatory synapse types differs.

**Number and density of AMPAR subunits on AN-BC and AN-FC synapses**

We used FRIL, which enables the reliable detection and precise localization of target proteins at a nanoscale spatial resolution with a very high labeling efficiency (Tanaka et al. 2005; Masugi-Tokita et al. 2007), to determine the distribution and number of GluA1–4, GluA3, and GluA4 subunits at AN-BC and AN-FC synapses. The distribution and quantity of GluA1–4, GluA3 and GluA4 subunits within the IMP cluster areas of mouse AN synapses varied between synapse types.

**GluA1–4 (pan-AMPAR antibody labeling)**

The distribution of the gold labeling and expression levels of GluA1–4 in the mouse AN-BC and AN-FC synapses were very similar to the previous findings in rats (Rubio et al. 2014). The GluA1–4 AMPAR immunogold particles appeared to be homogenously distributed throughout the IMP clusters of all AN-BC synapses and a majority of AN-FC synapses (Figs. 1, 2, 3). However, in some AN-FC synapses, AMPAR labeling was not uniformly distributed over the IMPs, and in some cases, IMP cluster sub-regions entirely lacked labeling (Fig. 2b–d). Nevertheless, all IMP clusters of the AN-BC and AN-FC synapses displayed gold labeling for GluA1–4 (Table 3). As expected from the substantial variability in PSD areas, the number of gold particles per IMP cluster in both the AN-BC and AN-FC synapses was quite variable [coefficient of variation (CV) = 0.44 for AN-BC, CV = 0.59 for AN-FC]. In contrast, the average density of gold particles per IMP cluster was less variable (CV = 0.18 for AN-BC, CV = 0.39 for AN-FC).

The mean numbers of immunogold particles for AMPARs in the AN-BC and AN-FC synapses were similar (p = 0.2; Mann–Whitney U test; Fig. 6a; Table 4). The area of the IMP clusters was significantly smaller in the AN-BC synapses than in the AN-FC synapses (Table 2); thus, the average density of AMPARs was significantly increased by more than 1.5-fold in the AN-BC synapses (p < 0.0001, Mann–Whitney U test; Fig. 6a; Table 4).

Both the AN-BC and AN-FC synapses exhibited a strong positive correlation between the number of labeled AMPARs and the IMP cluster area, consistent with the possibility that the AMPAR density is constant across synapses with the same connection type (Fig. 6b; AN-BC: r = 0.90; AN-FC: r = 0.47) and the number of AMPARs in individual synapses strongly depends on the size of the AN synapses.

**GluA3**

The GluA3 immunogold particles appeared to be fairly uniformly distributed over the IMP clusters of all AN-BC synapses, but not the AN-FC synapses, which exhibited less gold labeling (Fig. 3). Nevertheless, all IMP clusters of the AN-BC and AN-FC synapses were gold labeled for GluA3 (Table 3). As expected from the substantial range of PSD areas analyzed for both synapse types, the numbers of gold particles for GluA3 were highly variable (CV = 0.63 for AN-BC, CV = 0.47 for AN-FC); the average densities of gold particles per IMP cluster were less variable (CV = 0.5 for AN-BC, CV = 0.35 for AN-FC). The IMP clusters of the GluA3 KO mice lacked GluA3 immunogold particles, confirming the specificity of the antibody (Fig. 5).

The quantitative analysis of the gold labeling indicated a 2.5-fold increase in the level of the GluA3 subunit in the AN-BC synapses compared with that in the AN-FC synapses (p < 0.0001; Mann–Whitney U test; Fig. 6a; Table 4). The average density was increased more than fivefold in the AN-BC synapses (p < 0.0001, Mann–Whitney U test; Fig. 6a; Table 4).

The correlation between GluA3 labeling with the IMP cluster area was substantially stronger in the AN-BC synapses (r = 0.70) than the AN-FC synapses (r = 0.25), suggesting that the GluA3 density was only constant across AN-BC synapses.

**GluA4**

The GluA4 immunogold particles appeared to be evenly distributed over the IMP clusters of all AN-BC and AN-FC synapses; however, less gold labeling was observed on AN-BC synapses (Fig. 3). Nevertheless, all IMP clusters of both synapse types were labeled for GluA4 (Table 3). As expected from the substantial variability in the PSD areas, the numbers of labeled GluA4 subunits were also highly variable.
among the AN-BC and AN-FC synapses (CV = 0.49 for AN-BC, CV = 0.48 for AN-FC); the average densities of the gold particles per IMP cluster were less variable (CV = 0.28 for AN-BC, CV = 0.27 for AN-FC). The IMP clusters of the GluA4 KO mice lacked GluA4 immunogold particles, confirming the specificity of the antibody (Fig. 5).

According to the quantitative analysis of the gold labeling, AN-FC synapses contain ~2.3-fold more GluA4 than AN-BC synapses (p < 0.0001; Mann–Whitney U test; Fig. 6a; Table 4). The average density of GluA4 subunits was also ~1.7-fold higher at the AN-FC synapses than the AN-BC synapses (p < 0.0001, Mann–Whitney U test; Fig. 6a; Table 4).

The correlation between GluA4 labeling with the IMP cluster area was substantially stronger in the AN-FC synapses (r = 0.62) than the AN-BC synapses (r = 0.32) (Fig. 6b), suggesting that the GluA4 density was only constant across AN-FC synapses.

**Intrasynaptic distribution of AMPAR subunits on AN-BC and AN-FC synapses**

We subsequently determined the two-dimensional distribution of AMPAR subunits along the postsynaptic plasma membrane using FRIL. The intrasynaptic distribution of AMPARs (GluA1–4), GluA3, and GluA4 relative to the border of demarcation was performed by defining six divisions (D1–D5, from the periphery to center, and outer rim division) as previously described (Budisanto et al. 2012, 2013).

**GluA1–4 (pan-AMPAR antibody labeling)**

At the AN-BC synapses, gold particles were preferentially distributed towards the center of the IMP cluster and decreased towards the peripheral edge of the PSD (Figs. 7, 8). The highest density of gold particles was observed in the central division (D5, 1470 particles/μm²), followed by the adjacent D4 division (907 particles/μm²) and the other divisions (p < 0.0001, one-way ANOVA). The most peripheral division of the IMP cluster (D1, 540 particles/μm²) and the outer rim division (365 particles/μm²) exhibited the lowest density (p < 0.0001, one-way ANOVA). Of the 21 IMP clusters analyzed, 17 clusters (81%) exhibited a peak distribution at the center.

At the AN-FC synapses, the distribution of gold particles for GluA1–4 within the IMP cluster was relatively homogenous (Figs. 7, 8). The differences in the densities between divisions within the IMP cluster were insignificant, with the exception of the outer rim division, which exhibited a significantly lower density (217 particles/μm², p < 0.01, one-way ANOVA).

**GluA3**

At the AN-BC synapses and as observed with the pan-AMPAR immunolabeling, the central division exhibited the highest concentration of gold particles (Figs. 7, 8). Significantly less gold labeling was observed towards the peripheral divisions of the PSD. The difference between the central density (D5, 1250 particles/μm²) and the adjacent D4 division (840 particles/μm²) was significant (p < 0.001, one-way ANOVA). The most peripheral division (D1) exhibited the lowest density (405 particles/μm²; p < 0.001, one-way ANOVA). Of the 22 IMP clusters analyzed, 15 clusters (68%) exhibited this peak central distribution of GluA3 immunogold particles.

At the AN-FC synapses, a low density of immunogold particles was observed throughout the subdivisions (Figs. 7, 8). Based on the intrasynaptic distribution, the gold particle density was apparently increased at locations away from the very center of the synapse.

**GluA4**

At the AN-BC synapses, the GluA4 labeling was relatively low in all divisions of the IMP cluster (Figs. 7, 8). According to the analysis of the intrasynaptic distribution, the density of gold particles was similar from the center to the outer rim of the IMP cluster (p > 0.5, one-way ANOVA), indicating a relatively homogenous distribution.
Fig. 6 Number and density of AMPAR subunits in IMP-clusters of AN-BC and AN-FC synapses. a Histograms show the average density and number of gold particles per IMP-cluster for AMPAR, GluA3, and GluA4 at AN-BC and AN-FC synapses. The number of AMPAR gold particles was similar for AN-BC and AN-FC synapses, although the density was higher for AN-BC synapses. The number and density of GluA3 gold particles were higher for AN-BC synapses than for AN-FC synapses. The number and density of GluA4 gold particles were higher for AN-FC synapses than for AN-BC synapses [Mann–Whitney U test (p < 0.0001****; p = 0.0001 ***)]. b Correlation of the number of gold particles and IMP-cluster area. Scatter-plots of the number of gold particles for GluA1–4; GluA3 and GluA4 vs. the IMP-cluster areas of AN-BC and AN-FC synapses (Spearman’s rank-order test).
At the AN-FC synapses, the intrasynaptic distribution of gold particles was relatively homogeneous within the central divisions (D5-D2) of the PSD (Figs. 7, 8). The most peripheral division (249 particles/μm²) and the outer rim division (66 particles/μm²) of the IMP cluster exhibited a significantly lower density than the other divisions ($p < 0.0001$, one-way ANOVA).

**Intrasynaptic distribution of AMPAR subunits on AN-BC synapses from WT and GluA3 KO mice**

The pan-AMPAR and GluA3 immunogold particles located within the IMP cluster were concentrated at the center of AN-BC synapses. We analyzed the intrasynaptic distribution of GluA1–4 and GluA4 immunogold labeling in
GluA3 KO mice to determine whether GluA3 contributed to this central distribution (Fig. 9).

**GluA1–4 immunolabeling**

The GluA1–4 labeling at the AN-BC synapses was relatively homogeneous in the GluA3 KO mice (Fig. 9). The differences in the densities between the 5 divisions of the IMP cluster were insignificant (p > 0.5, one-way ANOVA). The peripheral D1 and the outer rim divisions had the lowest density of gold labeling in the WT mice (D1: 477.4 particles/μm², outer rim: 200 particles/μm²; p < 0.01, one-way ANOVA). The intrasynaptic distribution of GluA1–4 on the AN-BC synapses from the WT and GluA3 KO mice exhibited a significant difference (p < 0.001; F: 36.34; simple two-way ANOVA). Based on these findings, AMPARs within the IMP cluster are not concentrated at the center of AN-BC synapses.

**GluA4**

The distribution of the GluA4 immunogold particles at the AN-BC synapses was similar in the GluA3 KO and WT mice (Fig. 9), suggesting that the lack of GluA3 does not affect the distribution of the GluA4 subunits within the PSD. Moreover, GluA4 does not compensate for the lack of GluA3.

**Discussion**

**Differences in AMPARs at AN synapses**

Not all presynaptic action potentials produce postsynaptic action potentials, and the filtering and selection of signals occur at synapses. The amplitudes and kinetics of postsynaptic responses are profoundly controlled by the subunit composition of postsynaptic receptors. The subunit composition may be differentially regulated at individual synapses to enable optimized information processing. Auditory information processing depends on the high frequency and precise timing of action potential firing of auditory relay neurons. Thus, GluA2-lacking AMPARs with high Ca²⁺ permeability and submillisecond gating kinetics are prominently expressed in many auditory relay neurons (Gardner et al. 2001).

Of the four AMPAR subunits, GluA3 and GluA4 have rapid gating kinetics. Using postembedding immunogold labeling, AN synapses on BCs and FCs of the cochlear nucleus were shown to contain GluA2/3 and GluA4 subunits (Rubio and Wenthold 1997; Wang et al. 1998; Whiting et al. 2009). However, these studies only analyzed the presence of AMPAR subunits at the synapse. Furthermore, the level of GluA3 expression was not determined, because the applied antibody recognized both GluA2 and GluA3 subunits. A quantitative analysis of the expression patterns of GluA3 subunits at the AN-BC and AN-FC synapses has not been performed. Specific antagonists for AMPAR subunits are not available; thus, the roles of GluA3 and GluA4 in mediating synaptic transmission at AN synapses are unknown. However, differences in the kinetics of the synaptic responses at the two synapses have been identified (Gardner et al. 1999, 2001). Synaptic transmission is extremely fast and reliable at AN-BC synapses, thus preserving the information contained in the timing of AN spikes (Gardner et al. 1999; Fujino and Oertel 2003). Synaptic transmission is significantly slower at AN-FC synapses than at AN-BC synapses (Gardner et al. 1999, 2001). This difference in kinetics may arise from a combination of the differences in AMPAR density, the central organization of AMPARs, and a GluA3- or GluA4-dominant subunit composition (Geiger et al. 1995). Our study provides novel
insights into the differences in kinetics between the GluA3 and GluA4 subunits.

Using FRIL and a pan-AMPAR (GluA1–4) antibody with superb labeling efficiency for functional AMPAR channels in FRIL samples (Tanaka et al. 2005), mouse AN synapses on BCs and FCs contain similar numbers of gold particles for GluA1–4, approximately 36 and 32, respectively. A similar number of gold particles for GluA1–4 was also identified in the rat (Rubio et al. 2014). Using specific antibodies against GluA3 and GluA4, AN-BC and AN-FC synapses were shown to contain both subunits. Interestingly, AN-BC synapses express more GluA3 subunits, whereas AN-FC synapses express more GluA4 subunits. The labeling efficiency of the GluA3 and GluA4 antibodies is unknown; however, on average, 25 gold particles for GluA3 were observed at AN-BC synapses, whereas only 10 gold particles were observed at AN-FC synapses. Ten GluA4 gold particles were observed at AN-BC synapses compared
with 22 particles at AN-FC synapses. Thus, GluA3 is the main fast gating AMPAR subunit present in the ultrafast AN-BC synapse, whereas GluA4 is the main subunit present in the AN-FC synapse, which is a slower synapse.

**Intrasynaptic distribution of GluA3 and GluA4 subunits**

The subsynaptic localization of AMPARs is an important aspect of PSD organization (MacGillavry et al. 2011, 2013). According to computational studies, the nanoscale organization of AMPARs and the location of glutamate release may impact the quantal synaptic response (Franks et al. 2003; Raghavachari and Lisman 2004). Postsynaptic AMPARs that directly oppose the presynaptic release site are likely to be exposed to high concentrations of glutamate. Therefore, the local density of receptors close to the release site would affect the strength of the postsynaptic response (Franks et al. 2003; MacGillavry et al. 2013; Lisman et al. 2007). However, the situation may not be this simple, because simulation studies also show that the AMPAR response and glutamate concentration are not linearly correlated and AMPARs that immediately face the release site may become saturated (Tarusawa et al. 2009). Moreover, glutamate release that occurs slightly offset from AMPAR clusters would also produce a sizable response because of the additive responses from immediately activated AMPARs (Tarusawa et al. 2009). The number of these intermediately activated AMPARs is also expected to decrease as the release site is shifted to the periphery of the synaptic specialization. The most efficient synaptic signaling would be achieved when presynaptic release and the postsynaptic AMPARs are concentrated at the center of synapses.

Live-cell super-resolution imaging studies of the PSD of cultured hippocampal neurons have indicated that receptors are not uniformly distributed within the PSD and are typically confined within the subsynaptic domains (Kerr and Blanpied 2012; MacGillavry et al. 2013; Tang et al. 2016). One study examined the organization of AMPAR subunits (GluA2/3, GluA4, and GluA2) at AN-BC synapses (Wang et al. 1998) and observed a homogenous distribution of receptors within the PSD. However, only one ultrathin section was analyzed per PSD. FRIL cannot capture the dynamics of AMPAR movement; however, the distribution of the molecules revealed by FRIL using fixed brain slices may be considered a two-dimensional snapshot of the AMPAR distribution at a specific time point. FRIL has been used to investigate the intrasynaptic distribution of AMPARs at the calyx of Held synapse on neurons from the medial nucleus of the trapezoid body (MNTB) (Budisantoso et al. 2013) and reticulo-geniculate synapses in the dLGN (Budisantoso et al. 2012). In these synapses, AMPARs are homogenously distributed throughout the PSD, and limited gold labeling is only observed at the most peripheral edge. Based on our data, the distribution of AMPAR gold labeling at AN-FC synapses is relatively similar to that at calyceal and reticulo-geniculate synapses, whereas the distribution at AN-BC synapses differs, because the immunogold particles concentrate at the center. A tendency toward central organization was also identified in the retinogeniculate synapse; thus, central organization and a homogeneous distribution may not be two completely separate states, and a continuum may exist in the organization of these receptors. The central organization was identified in 81% of the AN-BC synapses analyzed, suggesting that this peak distribution pattern is not random.

The intrasynaptic distribution of AMPARs may depend on the subunit composition. Here, AN synapses on BCs contained significantly higher levels of GluA3 (2.5-fold more gold particles) than AN-FC synapses. The GluA3 subunit may exhibit the central organization pattern. The signature central organization of the AMPARs distributed at AN-BC synapses is lost in the GluA3 KO mice. Regarding other CNS synapses, GluA3 expression levels at reticulo-geniculate synapses and the calyx of Held synapse have not yet been determined. However, according to an electrophysiology study, GluA4 is the main AMPAR subunit expressed at the calyx of Held synapse (Yang et al. 2011). The authors showed that the amplitude and frequency of the excitatory postsynaptic currents (EPSCs) are decreased in GluA4 KO mice, but not in GluA3 KO mice. GluA4-dominant synapses may exhibit a non-central and homogenous organization of AMPARs. A recent study in the hippocampus using postembedding immunogold labeling with a different antibody from the antibody used in the study of GluA3 in serial ultrathin sections also observed a more central localization of GluA3 the PSD than GluA1 (Jacob and Weinberg 2015). GluA1 was located closer to the edge of the synapse. Thus, high levels of GluA3 at a specific synapse may determine the organization of AMPARs within the center of the PSD.

**Functional implications of the intrasynaptic distribution**

AN-BC synapses are approximately half the size of AN-FC synapses and have a higher density of fast gating GluA3 AMPAR subunits, which appear to be concentrated at the center of the synapse. The kinetics of the AMPAR channels may be faster at smaller synapses, as fewer AMPARs would be exposed to low concentrations of glutamate. Based on simulations, the average rise time of AMPAR-mediated postsynaptic responses correlated with the synaptic area (Tarusawa et al. 2009); however, the effect was relatively small, and the postsynaptic response decay time was predominately determined by the deactivation kinetics of
AMPARs, which are independent of the glutamate concentration. Similar to AN synapses, retinogeniculate synapses are also approximately half the size of corticogeniculate synapses. However, the synapses contain similar numbers of AMPARs, implying that retinogeniculate synapses have a higher density of AMPARs. The response amplitude in synapses with a higher AMPAR density is expected to be slightly higher (Tarusawa et al. 2009). The variability of the response that arises from many other sources, such as synapses of the same size, may obscure this modest difference in the quantal response amplitude caused by the difference in the AMPAR density. Consistent with this hypothesis, the quantal responses from retinogeniculate and corticogeniculate synapses observed in electrophysiological recordings were not different (Tarusawa et al. 2009).

An important but unresolved question is whether the pre-synaptic release site is aligned with postsynaptic receptor clusters. In addition to the role of the intrasyaptic receptor distribution in the synaptic response per se, peripheral receptors may tend to exhibit lateral diffusion. Therefore, synapses that require high-fidelity transmission with no or low plasticity may concentrate receptors in the center of the synapse to avoid the loss of receptors due to lateral diffusion. Further characterization of the specific anatomical and molecular organization of presynaptic release sites and postsynaptic AMPARs within AN synapses on BCs and FCs is required to understand how the synapses are tuned for optimal central sound processing.

Acknowledgements The authors thank Mitsuru Ikeda for his technical assistance with sample preparation. We also thank Dr. T. Shigeoka and Dr. Y. Ishida for their gift of the UPATrap vector. This research was supported by Grants from the NIH (NIDC 013048 to M.E.R.) and the Biotechnology and Biological Sciences Research Council, UK (Grant BB/J015938/1 to E.M.).

Author contributions All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: M.E.R. Data acquisition: M.E.R. Data analysis and interpretation: M.E.R., K.M., N.K., Y.F., and R.S. Drafting of the manuscript: M.E.R. Critical revision of the manuscript for important intellectual content: M.E.R., K.M., N.K., Y.F., and R.S. Statistical analysis: M.E.R. Obtained funding: M.E.R. Technical support: N.K. and H.H. Study supervision: M.E.R. Generation of GluA3 and GluA4 KO mice: M.A. and K.S. Development and characterization of the anti-GluA1–4 antibody: E.M. Development and characterization of the anti-GluA3 and anti-GluA4 antibodies: M.I. Provided GluA3 and GluA4 KO mice: K.S.

Compliance with ethical standards

Conflict of interest E.M. is member of the Scientific Advisory Board of Hello Bio [http://www.hellobio.com].

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