Dynamics of the AMPA-Receptor Proteome in the Mammalian Brain

Jochen Schwenk,1,2 David Baehrens,1 Alexander Haupt,1 Wolfgang Bildl,1 Sami Boudkkazi,1 Jochen Roeper,3
Bernd Fakler,1,2,* and Uwe Schulte1,2,*
1Institute of Physiology, University of Freiburg, Hermann-Herder-Strasse 7, 79104 Freiburg, Germany
2Centre for Biological Signalling Studies (BIOSS), Albertstrasse 10, 79104 Freiburg, Germany
3Institute of Neurophysiology, Goethe University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany
*Correspondence: bernd.fakler@physiologie.uni-freiburg.de (B.F.), uwe.schulte@physiologie.uni-freiburg.de (U.S.)
http://dx.doi.org/10.1016/j.neuron.2014.08.044

INTRODUCTION

AMPAR-type glutamate receptors (AMPARs) are key elements of excitatory neurotransmission in the mammalian brain. At glutamatergic synapses, they mediate the fast excitatory postsynaptic current and are involved in a variety of fundamental processes ranging from synaptogenesis to activity-dependent plasticity (Cull-Candy et al., 2006; McAllister, 2007; Raman and Trussell, 1992; Sah et al., 1990; Silver et al., 1992). Notwithstanding, many of these processes show pronounced variability across brain regions, types of neurons, and/or even synapses as a result of distinct functional properties, distinct processing, and/or trafficking and distinct susceptibility of the respective AMPARs to regulatory modifications (Anggono and Huganir, 2012; Derkach et al., 2007; Geiger et al., 1995; Hanley, 2010; Isaac et al., 2007; Lu and Roche, 2012; Moebacher et al., 1994; Trussell, 1999). Likewise, the properties and characteristics of AMPARs change during postnatal development when, upon formation of synapses and organization of neuronal circuits, the receptor channels are adapted to refined tasks and cellular requirements (Bredt and Nicoll, 2003; Brill and Huguenard, 2008; Feldman et al., 1999; Peng et al., 2009; Song and Huganir, 2002; Zhang et al., 2008). These dynamics and functional diversity of the AMPARs must reflect context-specific modulation and subunit composition of the receptors.

Native AMPARs in the mammalian brain are macromolecular complexes assembled from a total of more than 30 different constituents, mostly transmembrane proteins of different classes and secreted proteins (Schwenk et al., 2012; summarized in Table S1 available online). The entirety of these building blocks, also termed proteome, define both the diversity of the AMPARs and their layered architecture that is shared among all receptor channels (Schwenk et al., 2012). Accordingly, AMPARs consist of a defined core and a more variable periphery. The receptor core is formed by tetramers of the pore-forming GluA1–4 proteins (Hollmann and Heinemann, 1994; Seeburg, 1993; Sobolevsky et al., 2009) and up to four members of three distinct families of membrane proteins: the transmembrane AMPAR regulatory proteins (TARPs, γ2, γ3, γ4, γ5, γ7, γ8; Milstein et al., 2007; Tomita et al., 2003), the cornichon homologs 2 and 3 (CNH2, 3; Schwenk et al., 2009), and the GSG1l protein (Schwenk et al., 2012; Shanks et al., 2012). The periphery of the receptors is built from a set of transmembrane and/or soluble proteins that include CKAMPs 44 and 52 (von Engelhardt et al., 2010), PRRTs 1 and 2, and LRRT4 as well as four isoforms of the MAGUK family, Noelins1–3 and C9orf4/FRRS1l (Schwenk et al., 2012; see also Table S1). These proteins assemble with the core subunits either through direct binding or through interaction(s) with each other (Schwenk et al., 2012). Their distinct combinatorial architecture defines the function of the AMPARs. The inner core largely determines the biophysical properties of the receptors, including agonist-triggered channel gating, ion selectivity, and permeation, or block by polyamines, and influences their biogenesis and protein processing (Bats et al., 2007; Chen et al., 2000; Cho et al., 2007; Coombs et al., 2012; Kato et al., 2010a; Schwenk et al., 2009; Soto et al., 2007; Soto et al., 2009; Studniarczyk et al., 2013; Tomita et al., 2005). The periphery of the AMPARs seems to be involved in various aspects of synapse physiology (Cantallops et al.,

SUMMARY

Native AMPA receptors (AMPARs) in the mammalian brain are macromolecular complexes whose functional characteristics vary across the different brain regions and change during postnatal development or in response to neuronal activity. The structural and functional properties of the AMPARs are determined by their proteome, the ensemble of their protein building blocks. Here we use high-resolution quantitative mass spectrometry to analyze the entire pool of AMPARs affinity-isolated from distinct brain regions, selected sets of neurons, and whole brains at distinct stages of postnatal development. These analyses show that the AMPAR proteome is dynamic in both space and time: AMPARs exhibit profound region specificity in their architecture and the constituents building their core and periphery. Likewise, AMPARs exchange many of their building blocks during postnatal development. These results provide a unique resource and detailed contextual data sets for the analysis of native AMPAR complexes and their role in excitatory neurotransmission.
2000; Chen et al., 2000; Hussain et al., 2010; Siddiqui et al., 2013; von Engelhardt et al., 2010; Zhu et al., 2002). It must be emphasized, however, that most of these implications have not yet been mechanistically resolved and that the majority of the periphery-forming AMPAR constituents currently lack annotations of defined primary cellular functions.

The building blocks of native AMPARs were determined by proteomic analyses that used membrane preparations from whole rodent brains as input and comprehensively cataloged the AMPAR proteome, rather than defining regional or cell-type-specific differences (Schwenk et al., 2012). As yet, information on the expression pattern of individual AMPAR constituents across different brain regions is available only through data from immunohistochemistry and/or in situ hybridization. However, these data do not provide information on the subunit composition of the AMPAR complexes in distinct brain regions or types of neurons.

Here we applied high-resolution functional proteomic analyses (Schulte et al., 2011; Schwenk et al., 2010, 2012) to resolve the composition of AMPARs in defined regions of the rat brain and at distinct stages along the postnatal development. The results provide a roadmap for investigating the functional significance of the individual constituents of the AMPAR proteome and for research directed to unravel the molecular mechanisms underlying the complex cell physiology of AMPARs in the brain.

RESULTS

Distribution and GluA Composition of AMPARs across the Brain Regions

For profiling the spatial dynamics of the AMPAR proteome, we performed affinity purifications (APs) on solubilized membrane fractions from distinct brain regions that were obtained by macroscopic dissections dividing the adult rat brain into olfactory bulb, cortex, striatum, thalamus, hippocampus, brainstem, and cerebellum (Figure S1). All APs applied the same set of anti-GluA antibodies (ABs; Experimental Procedures) that recovered the entire pool of the GluA1–4 proteins; for solubilization, the previously established buffer systems CL-47 and CL-91 were used that both preserve high-molecular weight complexes but differ in their solubilization efficiency and ability to recover AMPARs of distinct subunit composition (Schwenk et al., 2012). The eluates of all APs were analyzed by high-resolution nanoflow mass spectrometry (nano-LC MS/MS), which, together with the calibration standards for quantification of peptide signal intensities (QconCAT proteins), provided data on the molecular abundance of all individual constituents of the AMPAR proteome (Schwenk et al., 2012).

These data on protein quantities were first used to determine total amounts, densities, and pore composition of the AMPARs across the aforementioned brain regions. As illustrated in Figure 1, the density of AMPARs which was determined as the total...
amount of the pore-forming GluA1–4 proteins in equivalent amounts of total membrane was distinct: highest densities of AMPARs were found in the hippocampus, followed by cerebellum (86% of hippocampus) and cortex (58%). The lowest densities were observed in thalamus and brainstem, where the densities of the AMPARs were as low as 10% of the hippocampal value (Figure 1). When related to the weight of the dissected tissue samples (Figure S1), the densities defined the amount of the AMPARs in a given brain region. Accordingly, about 50% of all AMPARs in the adult rat brain are found in the cortex, while hippocampus and cerebellum together harbor another 40%. The AMPARs in the remaining regions amount to less than 10% of all AMPAR assemblies in the adult rat brain (Figure 1).

Analysis of the molecular abundances determined for the individual GluA1–4 subunits showed that the composition of the receptor pore(s) varies throughout the brain, displaying considerable regional specificity (Figure 1). Thus, in the hippocampus, GluA1 and GluA2 are the most abundant pore-forming subunits, making up ~80% of the entire GluA pool, similar to reports from genetic analyses (Lu et al., 2009), while in the cerebellum these subunits amount to only 25%. Conversely, in the cerebellum the amount of GluA4 alone is as high as 64% (Figure 1). The GluA profiles determined for the other brain regions are intermediate between these two extremes, with cortex, thalamus, striatum, and olfactory bulb being closer to the hippocampus, and brainstem more resembling the cerebellum. It is noteworthy that the AMPARs in cortex and striatum (about half of the AMPARs in rat brain) exhibit very similar GluA profiles with GluA2 as the predominant subunit (~45%), roughly equal amounts for GluA1 and GluA3 (between 21% and 27%) and a minor portion of GluA4 (~8%; Figure 1).

**Spatial Distribution of All Constituents of the AMPAR Proteome**

In a next step, the analyses were extended to the non-pore-forming constituents of the AMPAR proteome, the additional building blocks of the receptors’ core and those of their periphery. The respective data on molecular abundances determined in the distinct brain regions at two solubilization conditions are summarized in Figure 2 (color-coded heat map) and in Table S1 (absolute values for the molecular abundance) together with the data on GluA1–4.

As indicated in either presentation, the values of the protein amounts determined for the entire proteome of the AMPARs extended over a broad range, spanning almost four orders of magnitude, similar to previous results of APs from whole brain (Schwenk et al., 2012). Moreover, the data presented two main observations: first, all 34 proteome constituents displayed considerable variations in their molecular abundances across the distinct brain regions (rows in Figure 2). The values of the respective maximum/minimum ratio determined for any AMPAR subunit ranged from 5 (TARP γ-2) to more than 120 (PRRT1, mean value of ~35). Second, the AMPAR proteomes determined for the individual brain regions (columns in Figure 2) are largely different from each other. Both observations are independent of the solubilization buffer(s) used, although the abundance values determined for some of the proteome constituents are influenced by the detergents, likely as a result of the particular biochemical properties of the respective AMPAR assemblies (detailed in Schwenk et al., 2012). Thus, Neuritin, Noelins1–3, or Brorin exhibit higher abundance values with CL-91, while TARP γ-8, ABHDs 6 and 12, and CPT1C were seen at significant amounts only under low-stringency solubilization with CL-47 (Figure 2).

**Composition of AMPAR Complexes in Distinct Regions of the Brain**

The abundance data presented above (Figure 2; Table S1) illustrate the spatial dynamics of the AMPAR proteome, without immediately linking the absolute protein amounts to specific AMPAR assemblies. For this purpose, the abundance values were related to functional receptors via normalization to the total number of GluA tetramers using (\(\sum\)) of GluA1–4 abundances/4 as a normalization factor. The resulting “normalized molecular abundance” values (Figures 3, 4, and 5) indicate the “average stoichiometry” of any proteome constituent across the entire pool of AMPARs expressed in a given brain region. Alternatively, these values may be regarded as the “probability” for integration of a particular proteome constituent into an AMPAR complex.

In this sense, the normalized molecular abundances determined for the GluAs (Figure 3A, upper panel), TARPs, CNIHs, and GSG1l (Figure 3A, lower panel) illustrate the “average subunit composition” of the inner core of the AMPARs in the distinct brain regions and demonstrate their considerable diversity (Figure 3A; Table S1). This diversity is reflected by both the pore-forming GluA subunits, as detailed above, and by the members of the three families of auxiliary proteins. Thus, in the hippocampus TARP γ-8 and CNIH2, at about equal amounts, are the predominant constituents of the inner core, with only minor contributions from the other TARPs (γ-2, γ-3, γ-4, γ-7) and CNIH2 (Figure 3A, lower panel). In contrast, in the thalamus it is TARP γ-2 and γ-4 which, together with CNIH2, make up major parts of the inner core, and in the cerebellum, TARPs γ-2 and γ-7 are the predominant constituents of the receptor core, with only minor contributions from TARPs γ-8, γ-4, γ-5, and CNIH2 (Figure 3A). In cortex and striatum, the profiles of the inner core subunits resemble that of the hippocampus with TARP γ-8 and CNIH2 as the major constituents; the slight reduction for CNIH2 appears to be balanced by increased amounts of TARPs γ-2 and γ-3 (Figure 3A, lower panel). Moreover, comparison of the regional profiles for the GluAs and the auxiliary subunits showed that TARPs γ-8 and GluA4 are inversely correlated: the more GluA4 is assembled into the AMPAR inner core, the less TARP γ-8 is coassembled. Conversely, assembly of CNIH2 into the inner core appears to be determined by the sum of the two pore subunits GluA1 and GluA2 (Figure 3A).

In all brain regions, except for cerebellum and brainstem, the normalized abundances of the inner core constituents amount to values between 3 and 4, in line with the proposed architecture of up to 4 such constituents occupying two distinct pairs of binding sites (Schwenk et al., 2012). In cerebellum and brainstem, these numbers total to values of only ~2, indicating a different composition of the respective GluA4-dominated AMPARs (either only one pair of binding sites occupied or a significant number of AMPARs lacking TARPs and CNIHs).

The striking differences in the inner core architecture between hippocampus and cerebellum can be directly visualized by
AB-shift assays in which the binding of target-specific ABs adds mass to selected AMPAR assemblies, thereby allowing their separation on native gels (Figure 3B; Schwenk et al., 2012). Accordingly, the assays in Figure 3B that used a combined set of anti-GluA1 and anti-GluA2 ABs are particularly illustrative:

1. almost all hippocampal AMPARs contain several GluA1 and/or GluA2 subunits (leading to a pronounced shift), while these subunits only assemble into a fraction of cerebellar AMPARs (small shift);
2. in the hippocampus, GluA4 is mostly associated with GluA1/2, while in the cerebellum it either

Figure 2. Abundance Profiles of the Protein Subunits of Native AMPAR Complexes across the Rat Brain
Heatmap illustrating the molecular abundance of all constituents of the AMPAR proteome obtained in anti-GluA1–4 APs from membrane fractions prepared from the indicated brain regions and solubilized with either CL-47 (left panel) or CL-91 (right panel).
assemblies into heteromers with GluA1 or forms homomers; (3) the vast majority of hippocampal AMPARs, except a small portion of GluA4-containing receptors, contain CN1H2 and TARPs γ-8 and/or γ-2/3, while a considerable fraction of cerebellar AMPARs lacks these core constituents; and (4) CN1H2 and TARPs γ-2/3 assemble into distinct populations of AMPARs in both the hippocampus and the cerebellum (Schwenk et al., 2009). The results derived for the distinct assemblies of cerebellar AMPARs were further corroborated by serial APs performed with ABS against GluA4 and GluA1–3 and evaluated by quantitative MS analyses. Accordingly, GluA4 predominantly assembles into homomers or heteromers with GluA1, and, interestingly, these assemblies were mostly devoid of inner core subunits resolving the reduced value obtained above for the total amount of these subunits in the cerebellum (Figure S2). The remaining heteromeric AMPARs, including the abundant GluA2/3 assembly (Lambolez et al., 1992; Chen et al., 2000), were found to be decorated with the inner core proteins TARPs, CN1Hs, and GSG1l in about 4:1 stoichiometry (Figure S2).

Extension of the normalized molecular abundances to the remainder of the AMPAR proteome further emphasized the pronounced region specificity and diversity of the AMPAR assemblies observed with the inner core constituents (Figure 4). Overall the proteome constituents exhibit distinct profiles across the different brain regions (rows, Figure 4), as well as within the AMPAR assemblies of a given brain region (columns). In addition, there are several noteworthy observations: First, several constituents, including the secreted proteins Noelins1–3, Brorin, and Brorin-l, as well as PRRT1, are observed throughout the brain but show preference for a distinct region (PRRT1 (hippocampus), Noelins1–3 (thalamus and cerebellum), Brorin, Brorin-l (thalamus)). Second, C9orf4 (or FRRS1l) is an abundant component of AMPARs in virtually all brain regions with particularly high abundance values in thalamus (0.8), striatum (0.52), hippocampus (0.46), and cortex (0.36). Third, ABHDs 6 and 12, CPT1C, PORCN, and Sac1, a group of proteins with potential enzymatic activities, display very similar abundance profiles across all brain regions. Fourth, CKAMP44 appears prominently in cortex, striatum, and thalamus, different from the restricted expression previously reported for this AMPAR constituent based on in situ hybridizations (von Engelhardt et al., 2010).

Composition of AMPAR Complexes in Selected Subregions of the Brain

The proteome data obtained for the AMPARs from the different brain regions originated from heterogeneous populations of cells and synapses, and likely reflect a composite of different functional/cellular states. Consequently, the assembly profiles shown in Figures 3 and 4 represent averages over a diversity of AMPARs. In a next step, we aimed to estimate the extent of averaging effects and to enhance resolution and specificity of the AMPAR composition. For this purpose we analyzed the entire AMPAR proteome in membrane fractions prepared from source material that was sampled by “micropunches” from defined subregions of the brain containing a much smaller number of distinct types of neurons and synapses.

Figure 5A illustrates the normalized molecular abundances determined for the GluA1–4 proteins (right panel) from tissue punches (diameter ~200 μm) excised either from the stratum radiatum of the hippocampal CA3 region (left panel), from the molecular layer of the cerebellum (ML), or from the nucleus accumbens in the ventromedial striatum. In all three cases, the abundance values are in good agreement with the pore assemblies obtained for the AMPARs in the regional samples of hippocampus, cerebellum and striatum (Figure 1). Similarly, the inner core composition (Figure 5B) and the abundance values for a selected set of peripheral constituents (Figure 5C) obtained from the micropunches overall resemble the respective data from the macroscopic tissue samples (Figures 3 and 4). However, closer inspection using 2D plots of the abundance data (Figure 6) revealed differences between the AMPAR proteomes from micropunches and the regional proteomes. Thus, in any subregion the majority of the AMPAR constituents displayed deviations from the corresponding regional data, with distinct sets of constituents sticking out in either case (protein subunits marked in red in Figure 6). Moreover, all deviations were for the non-pore-forming subunits, while the relative abundance values of the GluAs were almost identical between micro and macro samples (gray symbols located on the equal abundance line; Figure 6).

In addition to the distinctions between regions and subregions, the 2D plots emphasized the marked differences between the AMPAR assemblies among subregions. This was most evident for the receptor complexes from hippocampal CA3 region and the cerebellar ML, where almost all AMPAR subunits differed in their average stoichiometry by one or two orders of magnitude, while only very few constituents showed about equal participation (Figure 6, lower right panel).

Preferred Coappearance of Proteome Constituents

The data described above primarily define the assembly of any individual proteome constituent into the AMPARs of a given brain region. The data may, however, also provide information on whether or not several constituents coassemble into the same AMPARs or preclude their mutual coassembly. This can be identified by correlation analysis.

We therefore applied the Pearson algorithm for pairwise linear correlation to the complete set of normalized abundance profiles obtained from both regions and subregions of the brain (see Experimental Procedures). The Pearson algorithm uses deviations from the mean abundance as a parameter (Figure 7B) and thus probes for coappearance of AMPAR subunits across the individual sets of proteomes. These analyses elucidated a large number of pairwise correlations (Table S2) that interconnected the entirety of the AMPAR proteome with respective correlation coefficients (R) ranging from 0.99 to 0.48 (Figure 7A). As illustrated by the dendrogram in Figure 7A, the Pearson analyses identified several groups of constituents that are tightly correlated (R values ≥ 0.84; Figure 7A; Table S2). These groups include (1) GluA4, TARPs γ-5 and γ-7, and Neuritin; (2) DLGs 1 and 3; (3) CN1H2, PRRT1, and TARP γ-8; and (4) Noelins1–3, Brorin, Brorin-l, and TARP γ-2; as well as (5) LRRT4, GSG1l, CKAMP44, GluA3, and DLG4 or (6) ABHDs 6 and 12 and PORCN.

More detailed analysis investigating the robustness of the R values (see Experimental Procedures) showed that the aforementioned correlations may be subdivided into two major...
Dynamics of the AMPAR Proteome in Space and Time

A

Legend on next page

B

(legend on next page)
categories: first, correlations whose R values are essentially based on the entire data sets (omission of the best-correlated data point (“leave-one-out,” bracketed values in Figure 7B) results in a minor reduction); and second, correlations that are dominated by one or very few data sets. Examples for both categories are illustrated in Figure 7B: CNIH2-PRRT1 and GSG1l-CKAMP44-GluA3-DLG4 (middle, lower panel) are representatives of the first category, while the correlation between Noelin2, Brorin, and Brorin-I shows the characteristics of the second category. On the cellular level, the distinct types of correlations may result from at least two different mechanisms—coassembly (based on biochemical preference) or co-expression (Figure 7A, inset). Accordingly, correlations with robust R values likely result from molecular coassembly of the respective proteins subunits into the same AMPARs, while correlations with labile R values may point toward coexpression as a cellular correlate, although coassembly cannot be ruled out.

As a note of caution, it should be emphasized that robust correlation between proteins is not equivalent to an exclusive partnering, nor are high R values per se indicative of robust coassembly across all brain regions. Thus, the amounts of CNIH2 exceed far those of PRRT1, indicating that coassembly of CNIH2 and PRRT1 may only be observed in a subpopulation of CNIH2-containing AMPARs. Similarly, the correlations between LRRT4, GSG1l, CKAMP44, GluA3, and DLG4 or between GluA4 and TARPs, and TARPs, γ-5 and γ-7 may well indicate preferred coassembly in distinct regions or types of cells, but do not indicate exclusive association between these proteome constituents.

In addition to positive correlations, the Pearson analysis also identified some pairs of negatively correlated proteome constituents, i.e., subunits that may disfavor coassembly into AMPAR complexes (Table S2). These include TARPs, γ-5 and γ-7, which are negatively correlated with GluA2 but display high R values for GluA4. Likewise, TARP, γ-8 that exhibits a high R value for correlation with GluA2 is negatively correlated with GluA4 (see also Figure 3A).

Dynamics of the AMPAR Proteome during Postnatal Development

In addition to regional differences, we investigated the variations of the AMPAR proteome in response to changes in the

Please cite this article in press as: Schwenk et al., Regional Diversity and Developmental Dynamics of the AMPA-Receptor Proteome in the Mammalian Brain, Neuron (2014), http://dx.doi.org/10.1016/j.neuron.2014.08.044
boundaries and cellular constraints” associated with postnatal development. To quantitatively resolve more subtle differences, we used triplicate sets of AMPAR APs from whole rat brains at postnatal days P7, P14 and > P28.

The resulting time-abundance profiles for the pore-forming GluA proteins (Figure 8A), as well as for the constituents of the inner core (B), and a selected set of peripheral constituents (C) of AMPARs affinity purified from membrane fractions prepared from 200 μM tissue punches. These micropunches were excised from slices of the hippocampal CA3 region (left panel in A; scale bar is 200 μM), the molecular layer of the cerebellum or the nucleus accumbens of adult rats.

mostly associated with an abundance minimum at P14 (CNIHs 2, 3, TARP γ-2, Noelin1 and 3, and ABHDs 6 and 12); or (4) a more or less continuous, and partly considerable, decrease (TARP γ-4, DLGs 3 and 4, Noelin2). Notably, a few AMPAR subunits remain nearly constant in their abundance over the entire postnatal period analyzed; this includes GSG1I, TARP γ-7, PORCN, and CPT1 (Figures 8B and 8C).

Similarly, the pore-forming GluA proteins also do not display major changes during the postnatal period; noteworthy, they fall into two pairs, GluAs 2 and 4 and GluAs 1 and 3, whose abundance-time profiles are almost antiparallel: the transient increase of GluA2 is compensated by an almost identical decrease in the amount of GluA4, and the decrease of GluA1 is compensated in both time course and protein abundance by GluA3 (Figure 8A).

DISCUSSION

This work presents a comprehensive and quantitative characterization of the protein building blocks of native AMPARs across the distinct brain regions and during postnatal development. The data demonstrate pronounced spatial and temporal dynamics of the AMPAR proteome and illustrate the marked differences between AMPARs present in various regions and developmental stages of the brain. The proteomic data provide the framework required for in-depth molecular analysis of AMPARs and their function in distinct cellular context; moreover, they will be instrumental for investigating the significance of the numerous AMPAR constituents that, so far, lack annotation of primary function(s).

Dynamics of the Subunit Composition of Native AMPAR Complexes

For thorough investigation of the dynamics of the AMPAR proteome we used a previously established approach combining multiepitope APs with high-resolution mass spectrometry and
QconCAT-based label-free quantification of protein amounts (Schwenk et al., 2012).

This approach offers several key properties decisive for the central goal of this study: First, the combination of unparalleled detection sensitivity and unequivocal identification of proteins (down to \(0.1\) femto-mole) with a broad dynamic range for quantification of their abundance (linear behavior over three to four orders of magnitude; Bildl et al., 2012) enabled comprehensive analysis of the AMPAR proteome in both macroscopic samples from brain regions (Figures 2, 3, and 4) and microsamples from tissue punches (Figures 5 and 6). Second, the use of calibration standards for MS-signal intensities (QconCAT) allowed for determination of normalized molecular abundance values, which in turn permit direct quantitative comparison of the individual proteome constituents (Pratt et al., 2006; Schwenk et al., 2012). Third, the use of a set of ABs specifically and effectively targeting the four GluA proteins guaranteed isolation and analysis of the entire pool of AMPARs in any given sample. Fourth, MS analysis enabled detection of phosphorylation sites on distinct constituents of the AMPAR proteome (Table S3).

Moreover, and particularly important, the use of the anti-GluA1–4 APs guarantees that all AMPAR constituents are analyzed simultaneously and that for any particular constituent only the portion assembled into AMPARs is considered (target restriction). In both of these aspects, our proteomic approach contrasts with other techniques used to characterize the regional expression patterns of AMPAR subunits, namely immunocytochemistry or in situ hybridization, which both depict their target(s) independent of their incorporation into AMPARs, and neither approach provides quantitative data on protein amounts. Additionally, both approaches are limited by the sensitivity of the probes or target-unrelated effects (such as crossreactivity). As a consequence, expression data available in the literature and publicly accessible databases may be incomplete or misleading, as observed for numerous constituents of the AMPAR proteome. For example, hippocampus-restricted expression reported for CKAMP44 (von Engelhardt et al., 2010), and missing or region-restricted expression indicated for CNIH3; TARP\(\gamma\)-3, TARP\(\gamma\)-4, TARP\(\gamma\)-7, and TARP\(\gamma\)-8; GSG1\(\lambda\); PORCN; Brorin; Brorin-I; LRRT4; or PRRTs 1 and 2 (Allen Brain Atlas; Ng et al., 2009). On the other hand, both immunocytochemistry and in situ hybridization may provide expression profiles down to cellular resolution which is beyond the ability of our proteomic approach and therefore prevents discrimination between AMPARs from distinct types of cells in a given tissue sample.
Implications of the Spatial and Temporal Dynamics of the AMPAR Proteome

Native AMPARs expressed in different (sub)regions of the brain are largely different in their subunit composition, or, in other words, distinct regions (Figures 2, 3, and 4), and, most likely, also distinct types of neurons (Figure 5) may be decorated with specific types of AMPARs. In addition, AMPARs undergo considerable changes in their subunit composition during postnatal development (Figure 8), thus adding a temporal component to the high degree of regional (spatial) specificity characterizing the assembly of the receptor channels.

This marked degree of spatial and temporal variation in molecular appearance may reflect the pronounced variations reported for the properties of native AMPARs in distinct brain regions and/or for distinct developmental stages. Moreover, the molecular diversity reflected in the regional proteomes may be envisaged to underlie the specific tuning and adjustments attributed to the AMPARs by the complex cell physiology in excitatory synapses including synaptogenesis, timely, and precise transduction of the electrical signal and synaptic plasticity. So far, however, defined functions have only been assigned to a subgroup of AMPAR constituents, members of the TARP family (summarized in Bats et al., 2013; Kato et al., 2010b; Milstein and Nicoll, 2008; Straub and Tomita, 2012) and the CKAMP44 protein (von Engelhardt et al., 2010). Very recently, the CNIHs were shown to determine the time course of excitatory synaptic transmission (Boudkkazi et al., 2014; Herring et al., 2013). In fact, CNIH2-containing AMPARs promoted a slow decay of synaptic currents in mossy cells (and CA1/3 pyramidal cells), while CNIH2-free AMPARs in neighboring interneurons displayed rapidly decaying currents (Boudkkazi et al., 2014). These findings of distinct properties linked to distinct AMPAR assemblies were obtained from neurons in the hilar region of the hippocampus where AMPARs overall presented with high expression levels of CNIH2 (10-fold excess over CNIH3; Figure 5). Similarly, it might be illuminating to investigate the functional significance of other constituents of the AMPAR proteome, e.g., by single or multiple knockdown/knockout approaches, in that region(s) or at those time points, where the target subunit(s) is most effectively assembled into AMPARs.

In fact, molecular guidance may be the greatest benefit of the results presented here. These results may serve as a roadmap for investigations of AMPAR-related processes in synaptic physiology, and they may prove particularly relevant for those...
proteome constituents for which no primary function has yet been determined.

**EXPERIMENTAL PROCEDURES**

**Biochemistry**

**Brain Dissections**

Whole brains of five adult rats were prepared after decapitation, and cerebellum, olfactory bulb, and brain stem were separated. The remaining brains were split into hemispheres; the entire cortex and the striatum were isolated from one hemisphere, while the other was used to dissect hippocampus and thalamus. Region samples (Figure S1) were pooled and shock frozen in liquid nitrogen; the remaining tissue was pooled and used to calculate the mass balance of the total brain. For investigations of the AMPAR proteome during postnatal development, whole brains were extracted from ten rats of postnatal stages P0–P3, P7, P14, and P28–P49. Micropunches (200 μm diameter for hippocampal CA3 region, 1,000 μm for cerebral ML and nucleus accumbens) were excised from conventional brain slices of ten animals using a commercial punch device (WPI, Sarasota, USA); two to five punches per slice and region were collected and pooled for further analysis.

**Membrane Preparations and Protein Solubilization**

Plasma membrane-enriched protein fractions were prepared from isolated brains and brain regions as described (Schwenk et al., 2012). Crude membrane fractions were prepared from tissue punches. Briefly, membrane vesicles of the postnuclear supernatant were homogenized in hypo-osmotic lysis buffer (10 mM Tris/HCl [pH 7.4], 1 mM EDTA). After a 30 min incubation on ice at constant shaking, membranes were pelleted by ultracentrifugation (200,000 × g). Pellets were resuspended in 20 mM Tris/HCl (pH 7.4). Membrane proteins were solubilized for 30 min at 4°C with buffers (at 1 mg protein/ml) CL-47 or CL-91 (Logopharm GmbH) supplemented with protease inhibitors. Nonsolubilized material was subsequently removed by ultracentrifugation (10 min at 125,000 × g).

**Affinity Purifications**

Solubilates (0.5 ml) were incubated with a mixture of anti-GluA antibodies (anti-GluA1 (Millipore, #AB1504); anti-GluA2 (NeuroMab, #75-002); anti-GluA2/3 (Millipore, #07-598); anti-GluA3 (Synaptic Systems, #182-203); anti-GluA4 (Millipore, #AB1508) and anti-GluA1, anti-GluA2, and anti-GluA4 (gift from M. Watanabe) that were immobilized on supporting material (magnetic Dynabeads Protein A/G) by crosslinking performed according to the manufacturer’s instructions (Life Technologies). The complete pool of AMPARs was pulled out by incubating 0.5 mg (for regional samples) or 150 μg (for micropunches) solubilized membrane proteins for 2 hr at 4°C with 25 μg of the immobilised set of antibodies. Untbound material was analyzed by western blot. After brief washing with the detergent buffer, bound proteins were eluted by adding SDS sample buffer. In all cases, isolated proteins were shortly separated on SDS-PAGE gels and silver stained prior to tryptic digest.

**Western analyses**

Western analyses were performed with anti-GluA1, anti-GluA2 (Millipore, MAB397), anti-GluA2/3, anti-GluA3, anti-GluA4, anti-TARP-2/4/8 (NeuroMab), and anti-CNIH2 ABs (Boudkkazi et al., 2014). AB-stained bands were visualized by anti-mouse, -goat, and -rabbit IgG-HRP (all Santa Cruz Biotechnology) and by ECL+ (GE Healthcare).

**BN-PAGE**

Two-dimensional BN-PAGE/SDS-PAGE separations were essentially done as previously described (Schwenk et al., 2012). The AB-shift experiments were performed by preincubation of solubilizes with saturating amounts of anti-GluA1 and anti-GluA2 ABs.
Mass Spectrometry
LC-MS/MS Analyses

MS analyses of trypsin in-gel-digested samples (split into a higher and a lower molecular weight slice) were carried out on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with a nanoelectrospray ion source (Proxeon Bio-systems, data in Figure 8) or on an Orbitrap Elite mass spectrometer with a Nanospray Flex Ion Source (both Thermo Scientific, all other experiments) using the following settings (separated by “/” for the respective machines): Precursor signals used for quantification were acquired with a target value of 500,000/1,000,000 and a nominal resolution of 60,000/240,000 (FWHM) at m/z 400 (scan range 370–1,700 m/z). Up to 5/10 data-dependent CID fragment spectra per scan cycle were acquired in the ion trap with a target value of 10,000 (maximum injection time 400/200 ms) with dynamic exclusion (repeat count 1; exclusion list size 500; repeat/exclusion duration 30 s; exclusion mass width ± 20 ppm), preview mode for FTMS master scans, charge state screening, monoisotopic precursor selection, and charge state exclusion (charge state 1) enabled. Vacuum-dried peptide samples were dissolved in 0.5% trifluoroacetic acid and loaded onto a trap column (C18 PepMap100, 5 μm particles; Dionex) with solvent “A” (0.5% acetic acid; 20 μL/min for 5 min). Sample loading and reverse-phase chromatography were performed on Ultima 3000 HPLCs (RSLCnano for Orbitrap Elite). Peptides were separated via a 10 cm C18 column (PicoTip Emitter, 75 μm, tip: 8 μm, New Objective, self-packed with ReproSil-Pur 120 ODS-3, 3 μm, Dr. Maiisch HPLC; flow rate 300 nL/min) and eluted by continuously increasing the percentage of solvent “B” (0.5% acetic acid in 80% acetonitrile; A/B gradient: 5 min 3% B, 60 min from 3% B to 30% B, 15 min from 30% B to 100% B, 5 min 100% B, 5 min from 100% B to 3% B, 15 min 3% B).

Database Search
LC-MS/MS data were extracted using “extract_msn.exe” (grouping tolerance 0.05; Thermo Scientific) for LTO Orbitrap XL and “msconvert.exe” (part of ProteoWizard; http://proteowizard.sourceforge.net/; version 2.2.3214) for Orbitrap trap. Peaks were searched against the following databases using the Mascot search engine (version 2.4.0; Matrix Science): UniProtKB/Swiss-Prot release 2013_11 and 2013_01, respectively (only mouse, rat, and human entries including P00761|TRYP_PIG, P00766|CTRA_BOVIN, and P00769|ALBU_BOVIN) for AP samples; UniProtKB/Swiss-Prot release 2012_11 (only Escherichia coli entries) + contaminants database cRAP from GPM (ftp://ftp.theom.org/fastas/cRAP/crap.fasta, 02/29/2012) + database containing the sequences of the calibration proteins used for QonCAT (Schwenk et al., 2012).

For preliminary searches peptide mass tolerance was set to ±15 or 25 ppm. After linear shift mass recalibration by a home-made script, tolerance was reduced to ±5 ppm for final searches. Fragment mass tolerance was set to ±0.8 Da. One missed tryptic cleavage and common variable modifications including S/T/Y phosphorylation were accepted for peptide identification. Significance threshold was set to p < 0.05. Proteins identified by only one specific MS/MS spectrum or representing exogenous contaminations such as keratins or immunoglobulins were eliminated.

Quantification of Proteins
Protein quantification was based on peptide m/z signal intensities integrated over time (peaks volumes) as described in Turecek et al. (2014) with an optimized procedure. Peptide features were extracted with MaxQuant (http://www.maxquant.org/; Cox and Mann, 2008) version 1.3.0 with integrated effective mass calibration. Features were aligned between different LC-MS/MS runs and assigned to the peptides identified by Mascot (retention time tolerance ±0.5 [LTQ Orbitrap XL: 1.0] or ±1.5 min and mass tolerance: ±1.5 or ±2 ppm for MS-sequenced and nonsequenced peptides, respectively) using an in-house developed software. If fewer than four peptides were available for quantification of an individual protein, the respective peak volume assignments were manually verified. The resulting peptide peak volume tables were used for protein quantification (molecular abundance). First, protein-specific peptides were ranked by their internal consistency (pairwise Pearson correlation), and subsequently each peptide peak volume was normalized to its maximum over all data sets resulting in relative peptide profiles. Relative protein profiles were scaled to best fit the peptide calibration values obtained by the label-free QonCAT method (Schwenk et al., 2012; Turecek et al., 2014) to obtain molecular abundance values (arbitrary units) for each protein. This procedure was optimized to obtain quantitative information with a high level of reliability, i.e., information based on a maximum of consistent peak volume data points. Normalized molecular abundance values (Figures 3, 4, 5, 6, and 8) were obtained by relating the molecular abundance values to the number of AMPA receptors (Figures 3, 4, 5, 6, and 8) though division by the factor of GluA1–4 abundances/4.

Pearson Correlation Analysis

Correlation of proteins was calculated as pairwise Pearson correlation coefficients (R) of abundance values across the entire data sets of macro- and microsamples. Starting with the R values for each pair of proteins, an agglomerative hierarchical clustering algorithm was applied to arrive at clusters of highest correlation. Using the nearest point method on distance measures (1–R), clusters were formed by single-linkage of protein pairs with highest correlation. To assess robustness of correlations, the minimal correlation coefficient was calculated by stepwise omission of individual data sets (one by one). The “leave-one-out” values were obtained by finally omitting the data set with the greatest impact on the R value. All computations and visualizations were carried out using in-house software using the environment of NumPy, SciPy, SciKit-learn, and Matplotlib in the Python programming language (https://www.python.org/).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and three tables and can be found with this article at http://dx.doi.org/10.1016/j.neuron.2014.08.044.

ACKNOWLEDGMENTS

We thank J.P., Adelman for thoughtful discussions and critical reading of the manuscript. The mass spectrometry data have been deposited to the ProteomexChange Consortium via the PRIDE repository (identifier, PXD001195). This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 746, TP16, and Fa 332/9-1) to B.F.

Accepted: August 19, 2014
Published: September 18, 2014

REFERENCES


