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Immunohistochemical detection by immersion fixation with Carnoy solution of particular non-*N*-methyl-D-aspartate receptor subunits in murine hippocampus

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Abstract

Immunoblotting analysis revealed heterologous distribution profiles of the non-*N*-methyl-D-aspartate (NMDA) receptor subunits, GluR1, GluR2 and GluR6, in membrane fractions prepared from murine discrete brain structures including hippocampus. In coronal sections fixed with paraformaldehyde (PA) solution after dissection from mice perfused with 4% PA, however, no marked immunoreactivity was detected to GluR6 subunit in any hippocampal subregions, with high immunoreactivities to both GluR1 and GluR2 subunits in the strata oriens, radiatum and lacunosum-moleculare of the CA1 and CA3 subfields and the stratum moleculare of the dentate gyrus in hippocampus. In coronal, sagittal and horizontal sections fixed with Carnoy solution after dissection from animals decapitated, by contrast, high immunoreactivity was additionally detected to GluR6 subunit in the stratum lucidum of hippocampus. The systemic administration of kainate not only resulted in marked neuronal losses along the CA1–CA4 pyramidal layers 1 week later, but also led to significant decreases in immunoreactivities to GluR1, GluR2 and GluR6 subunits in the CA1 and CA3 subfields on brain coronal sections prepared by immersion fixation with Carnoy solution. These results suggest that immersion fixation with Carnoy solution may be suitable and appropriate for reproducible and quantitative immunohistochemical detection of particular non-NMDA receptor subunits in murine hippocampus.

Keywords: Carnoy solution; Paraformaldehyde; GluR6 subunit; Immunohistochemistry; Stratum lucidum

1. Introduction

Glutamate (Glu) receptors (GluRs) are categorized into two major groups termed as ionotropic (iGluRs) and metabotropic (mGluRs) receptors on the basis of pharmacological, electrophysiological and biochemical differences (Nakanishi, 1992; Watanabe et al., 1994a; Niedzielski and Wenthold, 1995; Safieddine and Wenthold, 1997). The iGluRs construct integral ion channels selective for particular cations, whereas the mGluRs are coupled to G proteins for modulation of the production of intracellular signal messengers. The iGluRs are subdivided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (KA) receptor subtypes. In mouse brain, these iGluR subunits are cloned and designated as GluR1, GluR2, GluR3 and GluR4 subunits for AMPA receptors, GluR5, GluR6, GluR7, KA1 and KA2 subunits for KA receptors, and NR1, NR2A, NR2B, NR2C and NR2D subunits for NMDA receptors, respectively. Recent genetic studies have identified a member of a new class of the iGluR family, termed as NR3A and NR3B subunits for NMDA receptors (Ciabarra et al., 1995; Das et al., 1998; Nishi et al., 2001; Matsuda et al., 2002; Chatterton et al., 2002).

Accumulating evidence for the importance of subunit compositions in the functional diversity of ion channels assembled from different iGluR subunits is available in the literature. Of non-NMDA receptors, AMPA receptors are believed to mediate fast excitatory synaptic transmission through ion channels composed of homomeric and/or heteromeric assemblies from GluR1, GluR2, GluR3 and GluR4 subunits (Boulter et al., 1990; Keinänen et al.,

Abbreviations: AMPA, α-amino-3-hydoroxy-5-methyl-4-isoxazolee-propionate; CNS, central nervous system; DAB, 3,3′-diaminobenzidine tetrahydrochloride; GFAP, glial fibrillary acidic protein; Glu, glutamate; GluR, glutamate receptor; iGluR, ionotropic glutamate receptor; KA, kainate; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; PA, paraformaldehyde; PB, phosphate buffer; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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1990; Pellegrini-Giampietro et al., 1997). Receptor channels assembled from GluR1, GluR3 and GluR4 subunits are permeable to Ca^{2+}, however, whereas coexpression of GluR2 subunit leads to construction of recombinant channels rather impermeable to Ca^{2+} (Hollmann et al., 1991; Verdoorn et al., 1991), suggesting that GluR2 subunit is a determinant of Ca^{2+} permeability of AMPA receptor channels (Pellegrini-Giampietro et al., 1997). Similarly, GluR5 and GluR6 subunits could not only form functional homomeric KA receptor channels (Egebjerg et al., 1991; Herb et al., 1992; Sommer et al., 1992), but also create functional heteromeric KA receptor channels when assembled with KA1 and KA2 subunits that are both unable to construct functional homomeric channels (Herb et al., 1992; Howe, 1996; Swanson et al., 1996).

In situ hybridization and immunohistochemistry techniques reveal widespread and abundant distribution profiles of non-NMDA receptor subunits in vertebrate central nervous system (Rogers et al., 1991; Petralia and Wenthold, 1992; Martin et al., 1993). The fact that heterologous expression of particular subunits could lead to functional heterogeneity strongly suggests the importance of investigations on distribution profiles of each subunit for elucidation and clarification of mechanisms underlying different functionalities of iGluRs in the brain. In brain sections prepared from animals perfused with paraformaldehyde (PA) usually used in immunohistochemistry, however, artifactual high immunoreactivities are detected to NR2C subunit along the CA1–CA4 pyramidal and dentate granular layers of murine hippocampus (Yoneyama et al., 2003), where this particular subunit is absent from (Nakanishi, 1992; Watanabe et al., 1993; Wenzal et al., 1995). Moreover, pretreatment with proteases is required for reproducible detection of NMDA receptor subunits in hippocampal CA3 subfield on brain sections prepared from mice perfused with PA (Watanabe et al., 1998).

In this article, therefore, we have attempted to evaluate the adequacy and validity of fixation procedures usually and routinely employed in immunohistochemical analysis in order to detect immunoreactive non-NMDA receptor subunits on sections of murine brain using antibodies commercially available.

2. Materials and methods

2.1. Materials

Goat polyclonal antibodies directed against GluR1, GluR2, GluR3, GluR4, GluR5 and GluR7 peptides at each carboxyl terminus as well as GluR6 peptide at amino terminus were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). An anti-goat IgG antibody, anti-rabbit IgG and the avidin-peroxidase complex were obtained from Vector Laboratories (Burlingame, CA, USA). KA was provided by Ocean Product International (NS, Canada). Rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) was purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest purity commercially available.

2.2. Animals

The protocol employed here meets the guideline for animal experimentation of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques. Adult male Std-ddY mice weighting 25–30 g at 6 weeks after birth were purchased from a local supplier and housed in metallic breeding cages in a room with a light–dark cycle of 12 h and a humidity of $55 \pm 2\%$ at $25 \pm 1\,^{\circ}C$ with free access to food and water for at least 7 days before use. Animals were intraperitoneally injected with KA dissolved in phosphate-buffered saline (PBS) (pH 7.4) at 40 mg/kg as needed.

2.3. Immunoblotting procedures

Mice were killed by decapitation and brains were quickly removed for subsequent immersion in ice-cold homogenizing buffer at $2\,^{\circ}C$ for at least 5 min. Buffers and any other solutions used were sterilized each time before use by autoclave or filtration through a nitrocellulose membrane filter with a pore size of 220 nm. Each brain structure was then dissected and homogenized in 20 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 1 μg/ml of each protease inhibitor [(*p*-amidinophenyl) methanesulfonyl fluoride, benzamidine, leupeptin and antipain], using Physcotron homogenizer.

Homogenates were centrifuged at $100{,}000 \times g$ for 1 h and the resultant pellets were suspended in 10 mM Tris–HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecylsulfate (SDS), 0.01% bromophenol blue and 5% 2-mercaptoethanol at a volume ratio of 4:1, followed by boiling at $100\,^{\circ}C$ for 10 min. Each aliquot of 40 μg or 80 μg protein was loaded on a 7.5% polyacrylamide gel containing 0.1% SDS for electrophoresis at a constant current of 15 mA/plate for 2 h at a room temperature (SDS polyacrylamide gel electrophoresis (SDS-PAGE)) and then subjected to subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skim milk dissolved in 20 mM Tris–HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween-20, the membrane was reacted with one of the receptor subunit antibodies adequately diluted with the latter buffer containing 1% skim milk, followed by a reaction with an anti-goat IgG antibody conjugated with peroxidase. Proteins reactive with those antibodies were detected with the aid of ECL detection reagents, followed by exposure to X-ray films

for different periods to obtain films most adequate for subsequent densitometry. Protein content was measured by a Bio-Rad Protein Assay kit (Bio-Rad, CA).

2.4. Fixation by perfusion

Animals were anesthetized with sodium pentobarbital at 300 mg/kg, followed by transcardial perfusion with 0.9% saline and then with 4% PA in 0.1 M phosphate buffer (PB) (pH 7.4). Brains were removed, post-fixed for 2 h with 4% PA, Zamboni or Carnoy solution, and placed in 30% sucrose in PB at 4 °C overnight. Coronal sections were cut at a thickness of 10 μm in a cryostat at −20 °C, followed by storage in 0.1 M PBS at 4 °C for a period of up to 1 week.

2.5. Fixation by immersion

Animals were killed by decapitation, followed by freezing of whole brains on crashed dry ice and subsequent dissection of frozen coronal, horizontal or sagittal sections at a thickness of 10 μm with the aid of a cryostat at −20 °C. Sections were then fixed with Carnoy solution (ethanol:acetic acid = 6:1) at 4 °C unless otherwise indicated, followed by rinsing with PBS at room temperature and subsequent storage in 0.1 M PBS at 4 °C for a period of up to 1 week. Sections were also fixed with 10% formalin in place of Carnoy solution as needed.

2.6. Detection of immunoreactive subunits

Sections were rinsed by PBS at a room temperature, followed by incubation in PBS containing 1.5% horse normal serum and 0.1% Triton X-100 (blocking solution) at 25 °C for 1 h and then with individual primary antibodies against GluR1, GluR2 and GluR6 subunits appropriately diluted in blocking solution at 4 °C overnight. After being washed in PBS, sections were reacted with a biotinylated secondary antibody at a room temperature for 30 min. After being rinsed for 5 min with PBS three times, sections were placed in solutions containing avidin and biotinylated-peroxidase at 25 °C for 1 h according to the manufacturer's instructions. After being rinsed for 5 min with PBS twice, sections were washed with 50 mM Tris–HCl buffer (pH 7.5) once, and incubated for 5 min with 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris–HCl buffer (pH 7.5) containing 0.01% H_2O_2.

2.7. Nissl's staining

Mice were intraperitoneally injected with KA at a dose of 40 mg/kg, followed by decapitation 1 week after administration and subsequent freezing of whole brains on crashed dry ice for dissection of frozen coronal sections with a thickness of 10 μm in a cryostat. Frozen sections were thawed at room temperature, followed by immersion fixation with 10% formalin or Carnoy solution and subsequent incubation with 95% ethanol at 4 °C overnight. Sections were then treated with 0.05% lithium carbonate for staining with 0.1% cresyl violet.

2.8. Data analysis

The CA1 and CA3 pyramidal subfields and the dentate gyrus were individually selected manually within a fixed rectangular area of 310 μm × 420 μm or 100 μm × 210 μm from sections on the computer, followed by conversion into monochromatic data and subsequent quantitative calculation of the area under the curve using the Adobe Photoshop 5.5 software. Results are expressed as the mean ± S.E. and the statistical significance was determined by one-way ANOVA followed by Scheffe's multiple comparison procedure. Differences between treatments were considered statistically significant when $P < 0.05$.

3. Results

3.1. Regional distribution profiles

In order to assess the usefulness as well as the specificity of different antibodies commercially available for immunohistochemical detection, membrane preparations were prepared from various discrete central regions, followed by SDS-PAGE and subsequent reaction with antibodies directed against GluR1–GluR7 subunits adequately diluted (1:1000). No marked immunoblots were detected for GluR1 subunit at molecular weight positions other than 106 kDa, while GluR1 subunit was highly expressed at the corresponding molecular weight position of 106 kDa in hippocampus and cerebellum, with progressively lower expression in olfactory bulb, cerebral cortex, striatum, hypothalamus and midbrain (Fig. 1, top panel). The highest expression of GluR2 subunit was seen as a single immunoblot at a molecular weight position of 102 kDa in hippocampus among discrete structures examined, with moderate expression in other regions tested except medulla-pons (Fig. 1, middle panel). By contrast, GluR6 subunit was rather evenly expressed at a molecular weight position of 118 kDa in most central discrete structures except medulla-pons (Fig. 1, low panel). No marked immunoreactive blots were detected to these three iGluR subunits tested in medulla-pons under the conditions employed. However, no profound immunoreactivities were detected to other non-NMDA receptor subunits examined as single immunoblots at the corresponding molecular weight positions with numbers of nonspecific immunoblots in membrane fractions prepared from all discrete central structures tested under the conditions employed (data not shown). These included GluR3–GluR5 and GluR7 subunits. Therefore, subsequent immunohistochemical evaluations were done using antibodies with clear detection of their

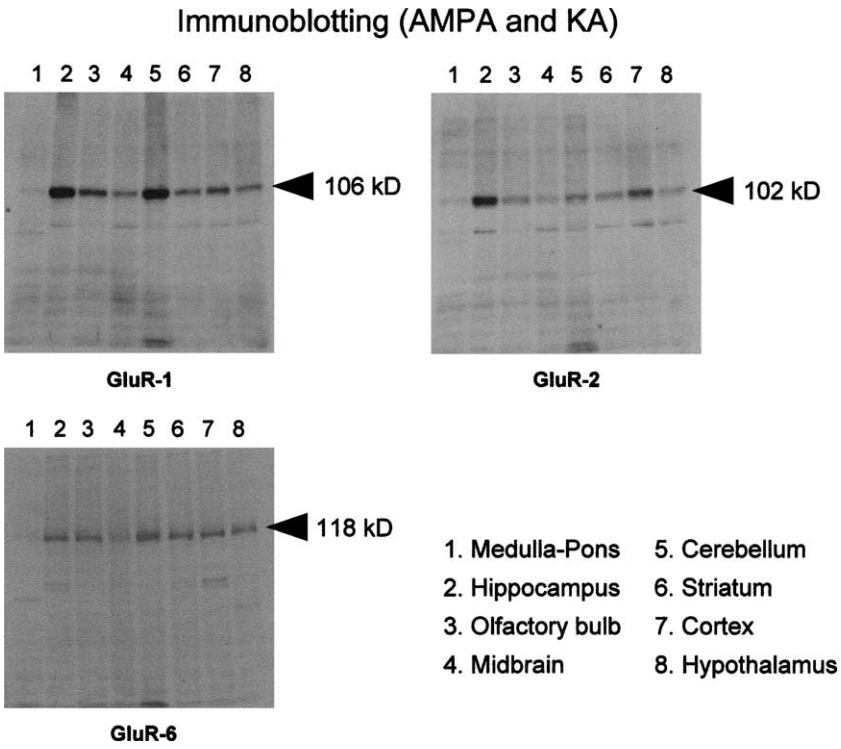

Fig. 1. Immunoblotting analysis of particular non-NMDA receptor subunits in mouse brain. Each discrete central structure was homogenized and centrifuged at 100,000 × *g* for 1 h. Resultant pellets were subjected to SDS-PAGE and subsequent immunoblotting assays using one of antibodies against GluR1, GluR2 and GluR6 subunits at a dilution ratio of 1:1000. Typical immunoblots are shown in the figure with similar results in three independent determinations. Black arrowheads indicate the molecular weight positions of the corresponding proteins.

immunoreactivities at the corresponding molecular weight positions on Western blotting analysis. These included antibodies against GluR1, GluR2 and GluR6 subunits.

3.2. Comparison of fixation procedures

Coronal brain sections were made from animals killed by perfusion with 4% PA solution under anesthesia, followed by successive incubations with normal blocking serum, primary antibody against a particular non-NMDA receptor subunit, biotinylated secondary antibody and avidin-biotin-peroxidase complex for conventional immunohistochemistry. In these sections, high immunoreactivities were detected with GluR1 and GluR2, but not GluR6, subunits in the strata oriens, radiatum and lacunosum-moleculare of the CA1 and CA3 regions, and the stratum moleculare of the dentate gyrus, respectively (Fig. 2a, upper panel). Expression of GluR6 subunit was not seen in any hippocampal regions, while no immunoreactive subunits were detected along the CA1–CA4 pyramidal and dentate granular layers.

Sections were then made from brains of animals killed by decapitation, followed by immersion fixation with Carnoy fixative and subsequent immunohistochemical detection of particular non-NMDA receptor subunits using the same antibodies. In these sections fixed with Carnoy solution, high immunoreactivities were detected to both GluR1 and GluR2 subunits in the strata oriens and radiatum of the CA1–CA3 subfields, with moderate immunoreactivities in the stratum lacunosum-moleculare of the CA1–CA3 regions and the stratum moleculare of the dentate gyrus (Fig. 2a, lower left and middle panels). No immunoreactive subunits were detected along the CA1–CA4 pyramidal and dentate granular layers. By contrast, relatively high expression was seen with immunoreactive GluR6 subunit in the region adjacent to the CA3–CA4 pyramidal layer (Fig. 2a, lower right panel). In the absence of primary antibodies, moreover, no marked immunoreactivities were detected in sections prepared from perfusion fixation with 4% PA solution and from immersion fixation with Carnoy solution (data not shown).

For confirmation of the usefulness of immersion fixation with Carnoy solution, horizontal and sagittal sections were made from brains of animals killed by decapitation for subsequent immunohistochemistry. In horizontal (Fig. 2b, upper three panels) and sagittal (Fig. 2b, lower three panels) sections, high immunoreactivities were again found with both GluR1 and GluR2 subunits in the strata oriens and radiatum of the CA1–CA3 subfields, with moderate immunoreactivities in the stratum lacunosum-moleculare of the CA1–CA3 regions and the stratum moleculare of the dentate gyrus. In contrast to sections prepared from perfusion fixation with 4% PA solution, relatively high expression of GluR6 subunit was again seen in the stratum lucidum of hippocampus on both horizontal and sagittal sections. No immunoreactive

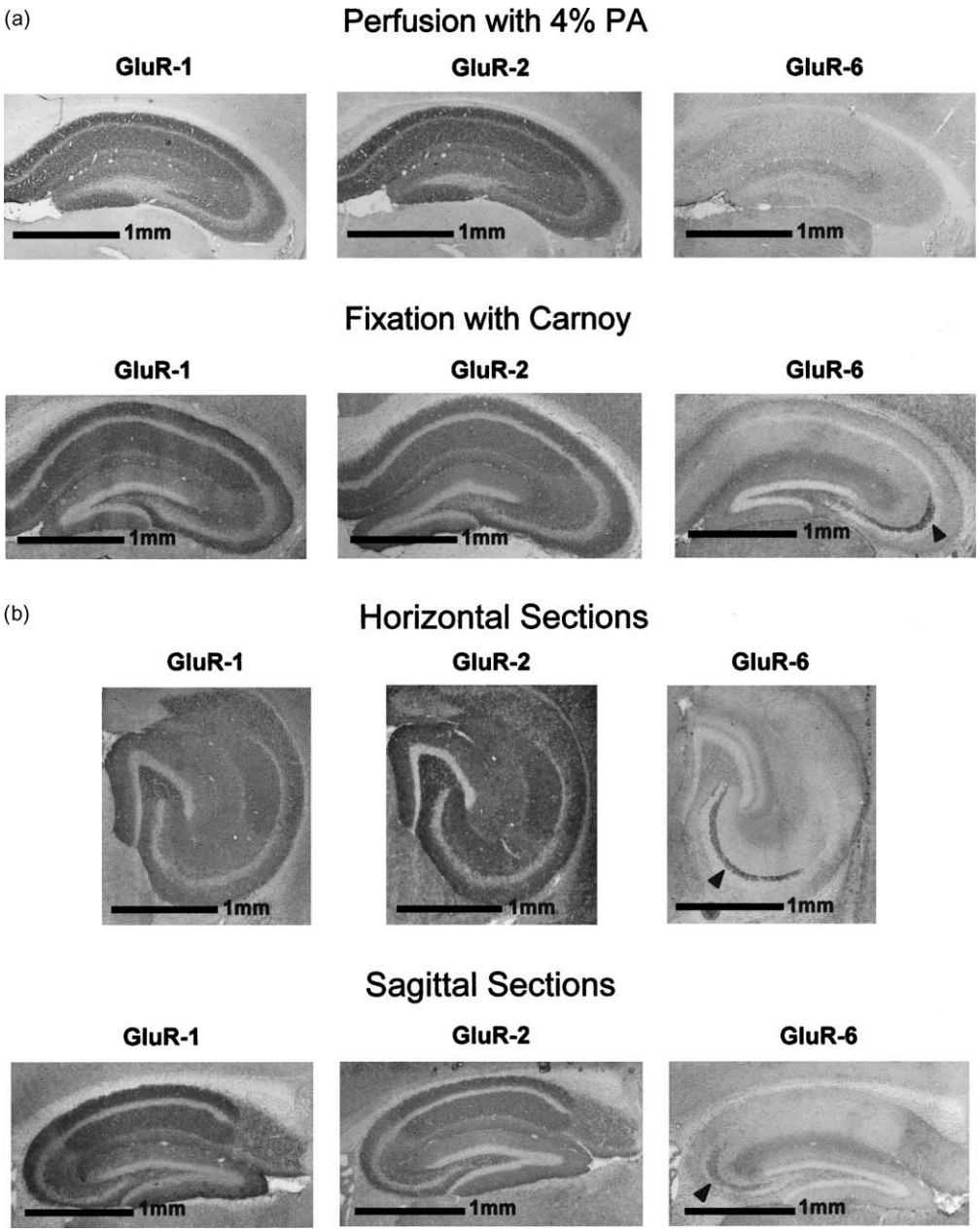

Fig. 2. Fixation of sections by different procedures. (a) Mice were killed by perfusion with 4% PA solution under anesthesia, followed by dissection of frozen coronal sections and subsequent incubation with one of antibodies raised against non-NMDA receptor subunits. Mice were also killed by decapitation, followed by dissection of frozen sections and subsequent immersion fixation with Carnoy solution (ethanol:acetic acid = 6:1) for incubation with one of antibodies raised against non-NMDA receptor subunits. (b) Mice were killed by decapitation, followed by dissection of frozen horizontal or sagittal sections and subsequent immersion fixation with Carnoy solution for incubation with one of antibodies directed against non-NMDA receptors subunits. Typical micrographs are shown in the figure with similar results in four independent animals. Black arrowheads indicate representative immunoreactivities to GluR6 subunit.

subunits were detected along the CA1–CA4 pyramidal and dentate granular layers as seen in coronal sections.

3.3. Other brain structures

In horizontal sections fixed by immersion with Carnoy fixative, both GluR1 and GluR2 subunits were widely distributed in the brain, with the highest immunoreactivity in hippocampus. In olfactory bulb, immunoreactivities were detected with GluR1 and GluR2 subunits in the glomerulus, while immunoreactive GluR6 subunit was highly localized in granule cell, ependyma and subependymal layers in addition to olfactory ventricle (Fig. 3a). Moreover, low immunoreactivity was detected for GluR6 subunit in glomerulus and external plexiform layer, with no marked immunoreactivities in the mitral cell layer.

In cerebral neocortex and caudate-putamen, relatively low immunoreactivities were detected for GluR1 and GluR2 subunits compared to hippocampus without immunoreactivity to GluR6 subunit (Fig. 3b). In the lateral septum, immunoreactivities were found for GluR1 and GluR2 subunits in the lateral septal nucleus (Fig. 3c). Immunoreactive GluR1 subunit was also detected in anterodorsal thalamic nucleus, moreover, while immunoreactive GluR2 subunit was seen in caudate-putamen and anteroventral thalamic nucleus. Low immunoreactivity was detected for GluR6 subunit in the lateral septal nucleus and caudate-putamen. In cerebellum, high immunoreactivities were detected with GluR1 and GluR-2 subunits in the molecular layer with no marked immunoreactive GluR1 and GluR2 in the granular layer (Fig. 3d). By contrast, immunoreactive GluR6 subunit was localized in the granule cell layer, but not in the molecular cell layer, of cerebellum.

3.4. Neuronal losses by KA administration

Mice were intraperitoneally injected with KA at 40 mg/kg, followed by decapitation 1 week after administration and subsequent dissection of frozen coronal sections for immunohistochemical analysis. Frozen sections were thawed at room temperature, followed by fixation with 10% formalin solution and subsequent staining with 0.1% cresyl violet as usual. Marked neuronal losses were seen in the CA1–CA3 subfields of hippocampal pyramidal cells within 1 week after the administration of KA, but not in the dentate gyrus of the granule cells, judging from intensities of staining by cresyl violet (data not shown). Compared to sections prepared from animals injected with saline alone, considerably high immunoreactivities were detected with the astroglial marker protein GFAP in the strata oriens and radiatum of the CA1 and CA3 subfields in hippocampus of animals injected with KA (data not shown).

In sections prepared by immersion fixation with Carnoy solution, marked losses of immunoreactivities were seen to GluR1 and GluR2 subunit in the strata oriens and radiatum of the CA1–CA3 subfields, but not in the stratum moleculare of the dentate gyrus, when analyzed 1 week after the administration of KA (Fig. 4a, upper and middle left and right panels). The administration of KA led to an almost complete loss of immunoreactive GluR6 subunit in the stratum lucidum of hippocampus (Fig. 4a, lower 2 panels). These sections were all subjected to quantitative analysis by subsequent densito-

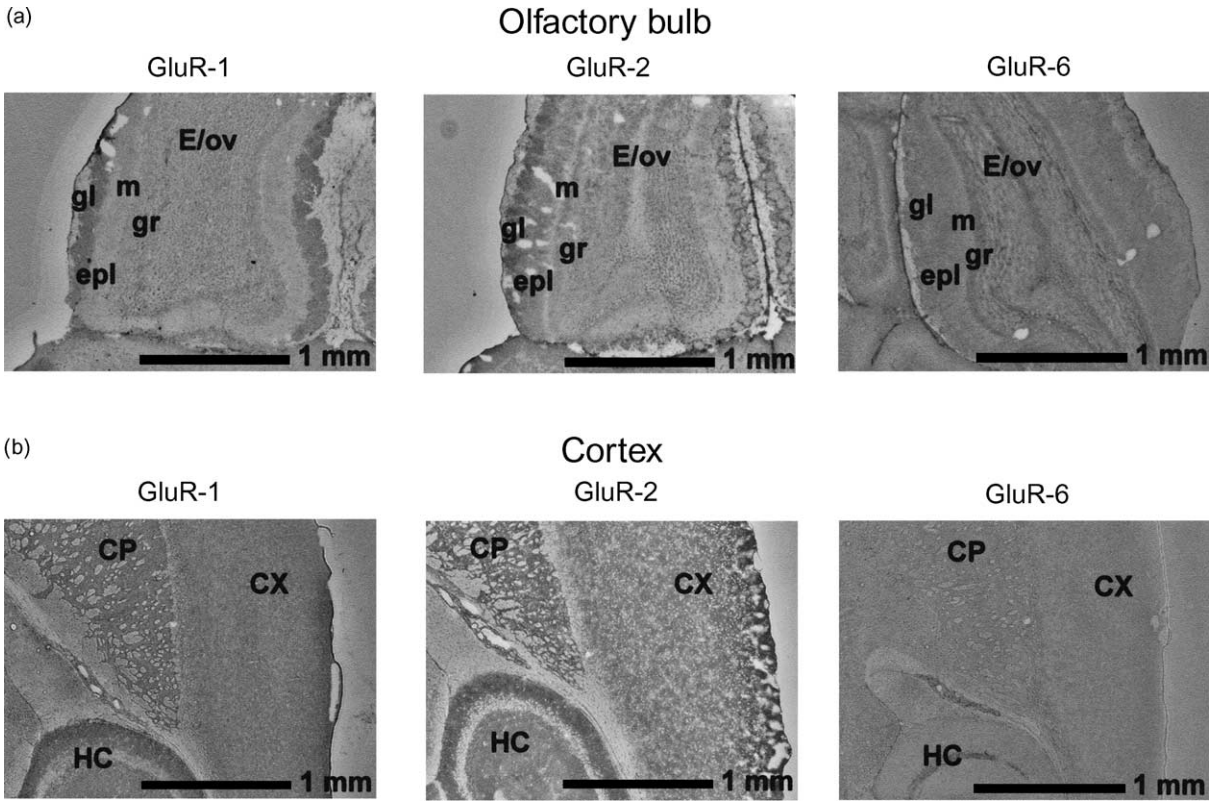

Fig. 3. Distribution profiles of non-NMDA receptor subunits in other central regions. Mice were killed by decapitation, followed by dissection of frozen horizontal sections and subsequent immersion fixation with Carnoy fixative (ethanol:acetic acid = 6:1) for incubation with one of antibodies directed against non-NMDA receptors subunits. Typical micrographs are shown for (a) olfactory bulb; (b) neocortex; (c) lateral septal and (d) cerebellum in figures with similar results in four independent animals. Abbreviations used: AD, anterodorsal thalamic nucleus; AV, anteroventral thalamic nucleus; CP, caudate-putamen; Cx, cerebral cortex; E, ependyma and subependymal layer; epl, external plexiform layer; gl, glomeruli; gr, granule cell layer; HC; hippocampus; LP, lateral septal nucleus; m, mitral cell layer; Mo, molecular layer; ov, olfactory ventricle; W, white matter.

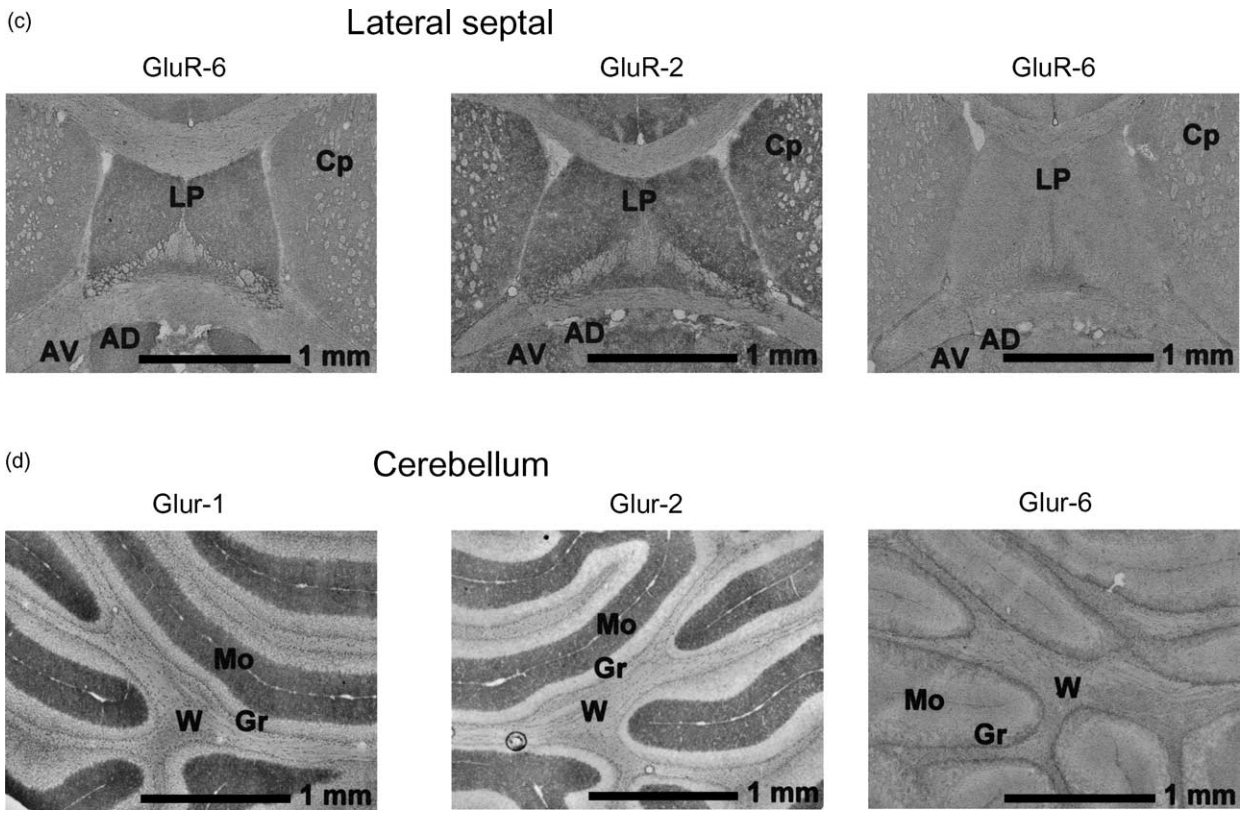

Fig. 3. (*Continued*).

metric determination of a density within a rectangular area of 310 μm × 420 μm in the individual CA1, CA3 and dentate gyrus subfields (Fig. 4b, upper left panel) as well as of 100 μm × 210 μm (Fig. 4b, lower middle panel). The administration of KA significantly decreased immunoreactivities to GluR1 and GluR2 subunits in the CA1 and CA3 subfields 1 week later, but not those in the dentate gyrus. The immunoreactive GluR6 subunit was significantly lost in the stratum lucidum, but not in the dentate gyrus, of hippocampus 1 week after the administration of KA.

4. Discussion

The essential importance of the present findings is that the KA receptor subunit GluR6 was highly localized in the stratum lucidum of the CA3–CA4 subfields in the hippocampus on murine brain sections prepared by immersion fixation with Carnoy solution. In contrast to rather ubiquitous expression in discrete brain structures on immunoblotting analysis, no immunoreactivity was detected to GluR6 subunit around the pyramidal and granular neuronal layers on brain sections prepared by perfusion with 4% PA often used for tissue fixation on conventional immunohistochemistry. Immunoreactivity to GluR6 subunit could be therefore crucial for evaluation of the suitability of tissue fixation procedures employed here for conventional immunohistochemical detection of non-NMDA receptor subunits in murine hippocampus. In fact, considerably high immunoreactivities are still detected with the NMDA receptor subunits NR2A or NR2B in the CA1 subfield of the pyramidal layer in brain sections prepared by perfusion with 4% PA of mutant mice knocked out of the corresponding subunits (Watanabe et al., 1998). In the present study, moreover, marked immunoblots were detected for GluR6 subunit at the molecular weight position of 118 kDa in hippocampal membranous preparations when analyzed on immunoblotting assays using an antibody identical to that used for immunohistochemical detection. These previous and present findings give support to the proposal that perfusion with 4% PA could be inappropriate as fixation procedures of brain sections for conventional immunohistochemical detection of particular non-NMDA receptor subunits in murine hippocampus.

The present distribution profiles of certain non-NMDA receptor subunits on brain sections prepared by immersion fixation with Carnoy solution, are fundamentally in good agreement with those revealed by autoradiographic analysis on frozen–thawed sections using [^{3}H]Glu (Greenamyre et al., 1985; Cha et al., 1988), [^{3}H]AMPA (Neilsen et al., 1988; Baudry et al., 1990) and [^{3}H]KA (Unnerstall and Wamsley, 1983) as a radioligand for detection of non-NMDA receptors. [^{3}H]Glu binding is localized in the stratum lucidum of rat hippocampus when determined in the presence of an agonist or an antagonist at NMDA receptors (Greenamyre et al., 1985), in particular, while [^{3}H]KA binding is abundant in the stratum lucidum (Unnerstall and

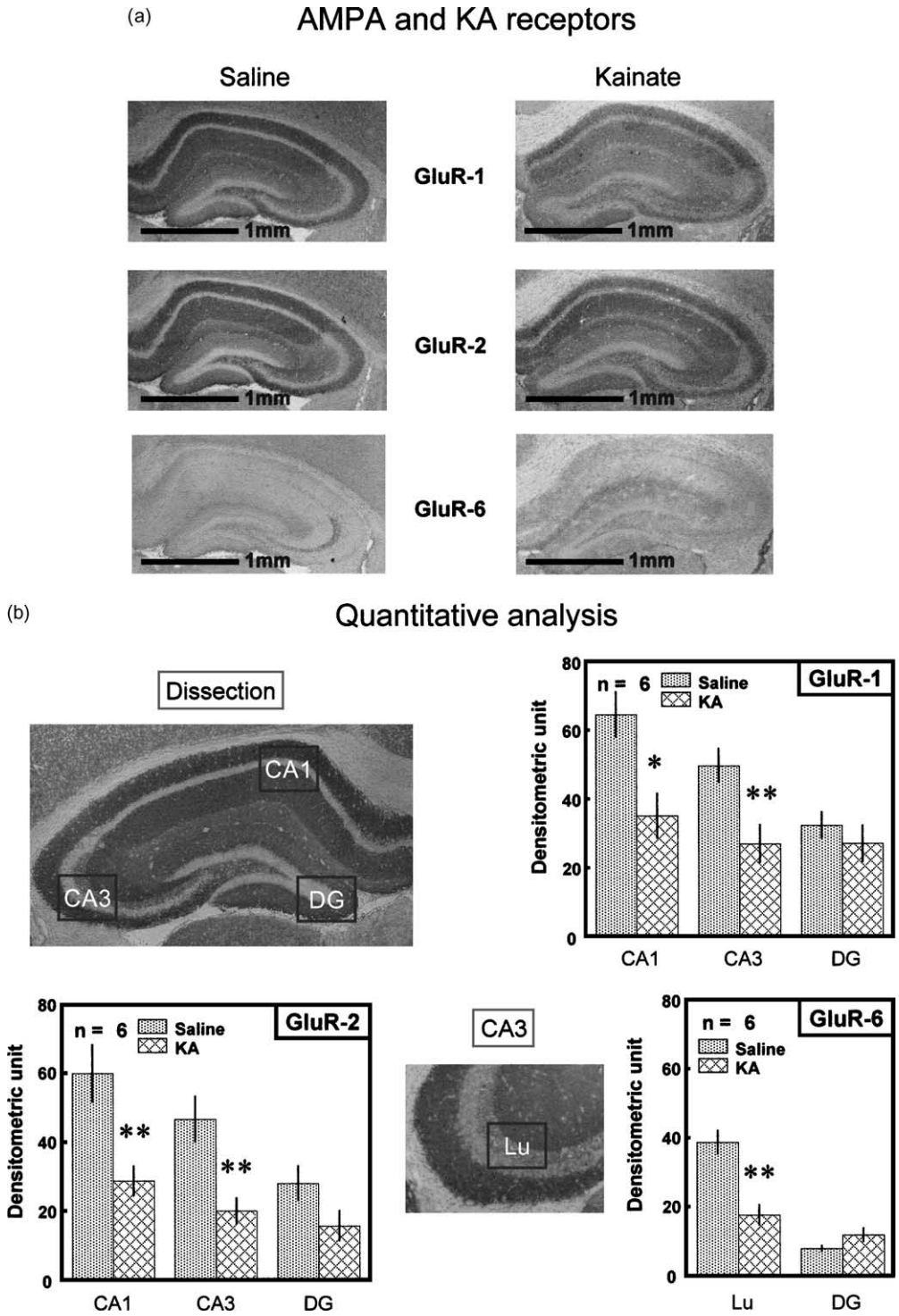

Fig. 4. Effects of administration of KA on distribution profiles of non-NMDA receptor subunits in mouse hippocampus. (a) Mice were intraperitoneally injected with KA at a dose of 40 mg/kg, followed by decapitation 1 week after administration for dissection of frozen coronal sections and subsequent immersion fixation with Carnoy solution (ethanol:acetic acid = 6:1). Sections were then incubated with one of antibodies raised against non-NMDA receptor subunits. Typical micrographs are shown in this figure. (b) Coronal sections were subjected to immersion fixation with Carnoy solution and subsequent incubation with one of antibodies raised against non-NMDA receptor subunits. Immunoreactive densities were quantified for both GluR1 and GluR2 subunits within a rectangular area of 310 μm × 420 μm in the CA1, CA3 and dentate gyrus as shown in the upper left panel. Similarly, densitometric determination was done within an area of 100 μm × 210 μm for GluR6 subunit in the stratum lucidum. Values represent the mean ± S.E. obtained in six separate animals. $^{*}P < 0.05$ significantly different from each control value obtained in animals injected with saline alone. Abbreviations: DG, dentate gyrus; Lu, stratum lucidum.

Wamsley, 1983). The present paper also deals with the first direct demonstration of localization of immunoreactive GluR6 subunit in granular, ependyma and subependymal layers as well as olfactory ventricle of olfactory bulb, and in granule cell layer of cerebellum, on immunohistochemical analysis. That no immunoreactivity was detected to the KA receptor subunit GluR6 in brain sections prepared from animals perfused with 4% PA, argues in favor of the usefulness and/or superiority of immersion fixation of brain sections with Carnoy solution rather than perfusion fixation with 4% PA for conventional immunohistochemical analysis on distribution profiles of particular non-NMDA receptor subunits in murine hippocampus.

Although the exact reason for these controversial results is not clear at present, one of the speculative explanations is that perfusion fixation with 4% PA solution (*aldehyde*) may be much more potent than immersion fixation with Carnoy solution (*alcohol*) in affecting antigenicity of the epitope through denaturation of corresponding antigenic proteins expressed in the cell surface. Perfusion with 4% PA could lead to artifactual deletion of immunoreactivities to GluR6 subunit expressed in the stratum lucidum through drastic alterations of antigenic properties of the epitope after potent denaturation in a particular situation. Indeed, pretreatment with proteases results in quantitative and qualitative alterations of distribution profiles of NMDA receptor subunits in murine hippocampus on brain sections prepared from animals perfused with 4% PA (Watanabe et al., 1998). In these sections treated with proteases, high immunoreactivities are detected with NR1 subunit in the strata oriens and radiatum of the CA1 pyramidal subfield as seen in sections prepared from immersion fixation with Carnoy solution (Yoneyama et al., 2003). In agreement with previous evaluation using sections treated with protease (Watanabe et al., 1998), moreover, relatively high immunoreactivities were found with NR1, NR2A and NR2B subunits in the stratum lacunosum-moleculare of the CA1 region, the strata oriens, radiatum and lacunosum-moleculare of the CA3 region, and the stratum moleculare of the dentate gyrus on brain sections prepared by immersion fixation with Carnoy solution (Yoneyama et al., 2003). Accordingly, immersion fixation with Carnoy solution seems to be preferable to perfusion fixation with 4%PA for evaluation of distribution profiles of particular non-NMDA and NMDA receptor subunits in murine hippocampus on conventional immunohistochemistry.

In our hands, the systemic administration of KA results in neuronal losses in both the CA1 and CA3 pyramidal layers without markedly affecting neuronal viability in the dentate granular layer of murine hippocampus within 1 week after administration (Kitayama et al., 1999). The present marked increases in immunoreactivities to GFAP are also suggestive of neuronal losses in the CA1 and CA3 pyramidal layers 1 week after the administration of KA. The findings that decreased immunoreactivities were seen with GluR1 and GluR2 subunits in the strata oriens and radiatum the CA1 and CA3 subfields and GluR6 subunit in the stratum lucidum, but not in the stratum moleculare of the dentate gyrus, therefore, give support to the validity of immersion fixation with Carnoy fixative for conventional immunohistochemical detection of particular non-NMDA receptor subunits in murine hippocampus over the tissue fixation by perfusion with 4% PA fixative usually and routinely employed in immunohistochemical analysis. The tissue fixation procedures should be carefully chosen for accurate and reproducible detection of non-NMDA receptor subunits in immunohistochemical studies. The fact that no marked changes were seen with distribution profiles of immunoreactive GFAP in the hippocampus between brain sections prepared by perfusion fixation with 4% PA fixative and immersion fixation with Carnoy fixative, makes it possible to speculate that the aforementioned artifactual deletion would occur with antigenic proteins expressed in the cell surface rather than those in the cytoplasm. Whether this idea could be extended to other membrane receptors, transporters, etc., however, remains to be elucidated in future studies.

It thus appears that immersion fixation with Carnoy solution would be better than perfusion fixation with 4% PA solution for accurate and reproducible detection of distribution profiles of non-NMDA receptor subunits in murine brain on immunohistochemistry. The pitfalls shown here would at least in part account for the possible paradoxical results between previous studies using different fixation procedures for certain membrane proteins.

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