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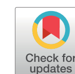
Antibodies raised against aldehyde-fixed antigens improve sensitivity for postembedding electron microscopy



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ABSTRACT

Background: Antibodies are one of the most important tools in biological research. High specificity and sensitivity of antibodies are crucial to obtain reliable results. Tissue fixed with glutaraldehyde (GA) is commonly used in electron microscopical investigations. The fixation and embedding routine in preparation of tissue for postembedding electron microscopy (EM) will mask and structurally alter epitopes, making antibody-antigen interaction inefficient, with low labeling intensities. One of the main factors in this regard is the use of GA as fixative.
New method: To alleviate these technical challenges, we immunized rabbits with antigen pre-fixed with GA. We hypothesized that the resulting antibodies would have stronger affinity to antigens that have been conformationally changed and denatured by GA, the way they are in fixed tissue.
Comparison with existing method and results: An initial screening with western blotting (WB) showed results consistent with our hypothesis. In-house antibodies raised against GA-fixed SNARE proteins SNAP-25 and VAMP2, binds more strongly to fixed proteins compared to non-fixed proteins, while the pattern is opposite with the commercially available antibodies raised against non-fixed antigens (standard antibodies). Quantitative postembedding EM of hippocampal synapses gave higher labeling intensities with anti-GA-SNAP-25 and anti-GA-VAMP2 compared to standard antibodies. Importantly, light microscopy (LM) and EM with our antibodies revealed stronger labeling of GA-fixed than formaldehyde (FH) treated brains.
Conclusion: Our results highlight the experimental potential of raising antibodies against GA-treated antigen to improve sensitivity of the antibodies for postembedding immunogold EM.

1. Introduction

Fixation of tissue is required to prevent postmortem decay, such as autolysis and putrefaction (Hladik, 2009). Fixation will also increase mechanical strength of the tissue as well as prevent bacterial colonization. In addition, the structure of the biological material is preserved so that they are very similar to that found in living organisms (Fox et al., 1985). The two most commonly used fixatives are GA and FA (Keiernan, 2000). Both are crosslinking fixatives and react with amino groups of protein and thus cross-link polypeptide chains (Sewell et al., 1984). GA is a larger molecule and diffusion through membranes is lower compared to FA. The advantage of GA is quick fixation and two aldehyde groups available that can act as a bridge between two proteins that are located more distantly from each other (Monsan et al., 1975). Weak fixatives with little or no GA provide optimum preservation of antigenicity and hence the strongest signal for immunocytochemistry. Strong fixatives, with a high concentration of GA, provide the best ultrastructure but may severely distort or mask target epitopes (Hayat, 2002).

The fixation and embedding routine in preparation of tissue for EM influences antigenicity, resulting in a markedly reduced labeling intensity and often increased background (Mathiisen et al., 2006). To overcome these technical challenges, we immunized rabbits with antigen pre-fixed with GA. We hypothesized that the resulting antibodies would have stronger affinity to antigens that have been conformationally changed and denatured by GA, the way they are in fixed tissue. SNARE proteins SNAP-25 and VAMP2 (also called synaptobrevin-2) were selected for immunization of the rabbits. Both proteins

Abbreviations: GA, glutaraldehyde; EM, electron microscopy; WB, Western blot; LM, light microscopy; FH, formaldehyde; IF, immunofluorescence; KO, knock-out; ON, overnight; SPB, sodium phosphate buffer; RT, room temperature; BB, blocking buffer solution; HSA, human serum albumin; WT, wild-type

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are members of a highly conserved family termed SNAREs (Fasshauer et al., 1998). These proteins together with syntaxin1 form a SNARE complex crucial for membrane fusion (Hussain and Davanger, 2011, 2015; Hussain et al., 2016, 2018). VAMP2 is associated with vesicles and termed a v-SNARE (vesicle-SNARE) while SNAP-25 is localized on plasma membrane and termed a t-SNARE (target-SNARE). The pairing of cognate v- and t-SNAREs between two opposing lipid bilayers drives membrane fusion and confers specificity to intracellular membrane trafficking (Hussain and Davanger, 2011; Hussain et al., 2017; Sollner et al., 1993). Crude antiserum was affinity purified against recombinant VAMP2 and SNAP-25 protein, both of them pre-fixed with GA. Anti-GA-SNAP-25 and anti-GA-VAMP2 antibodies were compared to similar commercially available polyclonal and monoclonal standard antibodies to determine whether they in fact more readily bind to fixed, denatured, protein than the standard antibodies. We further investigated sensitivity of GA-protein antibodies for GA- compared to FA-fixed brains. WB of synaptosomes, immunofluorescence (IF) labeling of neuronal cultures, LM of immunocytochemically stained brain sections, and post-embedding immunogold labeling of the hippocampus for EM were used to evaluate the performance of the antibodies.

2. Material and methods

2.1. Antibodies

GA-protein-antibodies: Anti-GA-VAMP2 and anti-GA-SNAP-25 were raised in rabbit. Recombinant VAMP2 and SNAP-25 proteins were fixed in 1.25% GA before being mixed with Freunds adjuvant and used for immunization. Crude antiserum was affinity purified against the same recombinant VAMP2 and SNAP-25 protein fixed with 1.25% glutaraldehyde (see below). Anti-GA-VAMP2 was used at 1:200 000 for WB, 1:300 for IF, 1:50 for LM, and 1:10 for EM. Anti-GA-SNAP-25 was used at 1:50 000 for WB, 1:100 for IF, 1:50 for LM, and 1:30 for EM.

Purchased polyclonal antibodies: Anti-VAMP2 (AbCam, Cambridge, UK, Cat#Ab70222) was used at 1:1500 for WB, 1:300 for IF and 1:350 for EM. Anti-SNAP-25 (AbCam, Cambridge, UK, Cat#41455) was used at 1:25 000 for WB, 1:300 for IF and 1:10 for EM. Anti-syntaxin1 (Alomone, Jerusalem, Israel, Cat#ANR-002) was used at 1:300 for LM.

Purchased monoclonal antibodies: Anti-VAMP2 (Covance, New Jersey, USA, Cat#MMS-616R) was used at 1:2000 for WB, 1:50 for IF and 1:10 for EM. Anti-SNAP-25 (Covance, New Jersey, USA, Cat#MMS-614R) was used at 1:5000 for WB, 1:300 for IF and 1:10 for EM.

The absolute concentration (e.g., µg/µl) of primary antibodies used in this study was unknown. Different antibodies, however, against the same antigen used at the same absolute concentrations will still have different affinities to the antigen. Thus, comparing different antibodies used at the same absolute concentrations will be of little value. In the current study, we have taken care to optimize the concentration for each antibody individually before comparing immunogold labeling intensities. We used series of dilutions in titration experiments. The optimal dilution was then selected based on specific labeling intensity of synaptic membranes combined with low non-specific labeling of mitochondria.

The following secondary antibodies were used: IgG coupled to 10 nm colloidal gold (British BioCell International, Cardiff, UK, Cat#AR14007) was used at 1:40 for EM. Goat anti-rabbit alkaline phosphatase (Sigma, MO, USA, Cat#A3687) and goat anti-mouse alkaline phosphatase (Sigma, MO, USA, Cat#A3562) were used at 1:10 000 for WB. Alexa fluor 488 (Thermo Fisher Scientific, Massachusetts, USA) donkey anti-rabbit (Cat#A21206) and donkey anti-mouse (Cat#A21202) were used at 1:1000 for IF. Biotynilated goat anti-rabbit (Abcam, Cambridge, UK, Cat#Ab64256) ready to use for LM. Streptavidin biotinylated horseradish peroxidase complex (GE healthcare, Buckinghamshire, United Kingdom, Cat#RPN1051 V) was used at 1:100 for LM.

2.2. Animals

Wistar male rats weighing 250–300 g were used for EM, 1–4 day old Wistar rats for primary hippocampal cultures, PVG male rats weighing 200–250 g for WB and LM. SNAP-25 knock-out (KO) mice (Washbourne et al., 2002) and VAMP2 KO mice (Schoch et al., 2001) were used to evaluate antibody specificity. Experimental protocols were approved by the Institutional Animal Care and Use Committee and conform to National Institutes of Health guidelines for the care and use of animals, as well as international laws on protection of laboratory animals, with the approval of a local bioethical committee and under the supervision of a veterinary commission for animal care and comfort of the University of Oslo. The animals were treated in accordance with the guidelines of the Norwegian Committees on Animal Experimentation (Norwegian Animal Welfare Act and European Communities Council, Directive of 24 November 1986-86/609/EEC). Every effort was made to minimize the number of animals used and their sufferings.

2.3. Antibody production and purification

2.3.1. Bacteria clones

We obtained bacteria clones for SNAP-25 and VAMP2 as a gift from Richard Scheller. A cDNA probe corresponding to nucleotides 21-302 from VAMP2 and 209-829 from SNAP-25 were cloned into pGEX-KG vector and expressed as fusion protein in E. coli.

2.3.2. Sequence analysis of plasmid

The bacteria clones for SNAP-25 and VAMP2 were grown on Luria-Broth agar plates containing ampicillin 50 µg/ml at 37 °C. Single colony was transferred to 2xYT-medium (bacto-tryptone 16 g/l, bacto yeast extract 10 g/l, NaCl 5 g/l) containing 20 µg/ml in the incubator at 37 °C and 220–250 rpm. The bacteria were grown to reach an absorbance A600 0.6–0.7. The standard protocol from Qiagen was used to isolate DNA. Restriction enzyme analysis and sequencing of DNA were performed to verify the inserted sequence.

2.3.3. Expression of GST-fusion protein

SNAP-25 and VAMP2 GST-fusion proteins were expressed and purified (Guan and Dixon, 1991). Bacteria were first grown in 10 ml 2xYT-medium with 50 µg/ml ampicillin in incubator at 37 °C and 220–250 rpm. Overnight (ON) culture was mixed with 1 L 2xYT media containing 20 µg/ml ampicillin and grown further in the incubator for 3 h at 37 °C (absorbance 0.6–0.7). Isopropyl BD-thiogalactoside was added to the culture to a final concentration of 0.4 mM and incubated at 37 °C for 2 h. The culture was centrifuged at 5000 rpm for 5 min at 4 °C using Sorvall RC 5B Plus centrifuge (SLA 1500 rotor). All the further steps were performed at 4 °C. The pellet was resuspended in 10 ml (1/100) of the following solution: 150 mM NaCl, 16 mM Na_2HPO_4 (pH 7,3), 4 mM NaH_2PO_4 (pH 7.3), 2 mM EDTA, 0.1% phenylmethylsulfonyl fluoride (resolved PMSF in ethanol), 5 mM Benzamidin and 1% Triton X-100. Lysozyme 2 mg/ml was added to the resuspension and incubated on ice for 10 min. Added 40 mM $MgCl_2$ and 10 µg/ml deoxyribonuclease and incubated on ice for 15 min. Sonicated with a Vibracell (Soniks and Materials Inc. Danbury, CT USA), 10 × 30 s, amplitude 70. Each tube contained 5 ml suspension. Centrifuged at 10 000 rpm for 10 min in a Sorvall RC 5B plus centrifuge (SS-34 rotor). Supernatant contained the expressed soluble protein.

2.3.4. Purification of GST fusion protein

A column with Glutathione Sepharose 4B (Amersham Biosciences) was used for purification of the protein. The supernatant was applied to the column. After flow through of the supernatant, the column was washed first with 20 ml sodium phosphate buffer (SPB) and then 10x bed volume of thrombin cutting buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, 0.1% B-mercaptoetanol). To cut the GST tag from the fusion protein, thrombin (5–10 µg/ml beads) was added to

the column and incubated ON at 4 °C. The eluated protein was collected. The column was then incubated with 1x bed volume thrombin cutting buffer for 10 min and the flow through was collected. This process was repeated 5 times. Glycerol was added to the eluated protein in a concentration of 20% on ice. The protein was stored at -80 °C.

2.3.5. Fixation of antigen with glutaraldehyde

Antigen was divided into 8 different tubes and GA added to a final concentration of 1.25%. The samples were shaken a few times until they got a yellow colour, and incubated at 4 °C for 2.5 h. Stock solution of 1 M glycine in phosphate buffer was added to the tubes to a final concentration of 200 mM and incubated for 1.5 h at 4 °C. Samples were then transferred to dialysis tubes. The tubes were incubated in a large beaker containing 0.2 M phosphate buffer ON on a magnetic stirrer at 4 °C. The next day, the buffer was changed three times with 6 h interval. The samples were stored at -80 °C.

2.3.6. Immunization of rabbit

Two rabbits were used for immunization of each antigen. 100 µg of antigen was mixed with complete Freund adjuvant to a total volume of 1 ml and injected intramuscularly. Two weeks later, the rabbits were injected with 100 µg (1 ml) of antigen mixed with incomplete Freund adjuvant. After 10 days, the antibody reached peak levels. Blood samples were incubated 1 h at room temperature (RT) and centrifuged at 3500 rpm for 10 min. The serum was stored at −80 °C.

2.3.7. Affinity purification of the antibodies

Affinity purification was based on affigel-15/affigel-10 (Amersham). The procedure was performed in a cold room with cold solutions. The affigel was stored in propanol at −20 °C. 10 ml affigel was mixed with acidic water on a shaker to allow swelling. A tube with 3 mg peptide and HEPES (50 mM, pH 8.0) was prepared, added to the affigel, mixed immediatelyand incubated ON. A column with the mixture of peptide and affigel was prepared. We used 10 column volumes of affigel to initialize the columns with starting buffer (50 mM Tris buffer, pH 7.4, 300 mM NaCl, 0.1% NaN_3 and 1 mM EDTA). Anti-serum was applied to the column. The flow rate was adjusted to 1 h. The column was re-initialized with washing buffer. The antibodies were eluted with two column volumes of elution buffer (100 ml: 10 ml 2 M Glycine-HCl pH 2.5, 5 ml 3 M NaCl, 1 ml 0.5 M EDTA, 0.5 ml 20% NaN_3). The eluate was collected directly in to 2 ml of 2 M HEPES buffer (pH 8). PMSF was added to the eluted IgG to a final concentration of 1 mM. The antibodies were stored at -80 °C.

2.4. Subcellular fractionation

Preparation of synaptosome and vesicle membrane fractions: Ten rats were decapitated, the brains were dissected out and submerged in ice-cold HEPES-buffered sucrose (0.32 M sucrose, 4.0 mM HEPES, pH 7.4) containing a protease inhibitor cocktail (Promega, Winconsin, USA). The tissue was homogenized in HEPES buffer with a glass-teflon homogenizer (900 rpm, 10–15 strokes) and centrifuged (800–1000 g, 10 min, 4 °C). The postnuclear supernatant, S1, was centrifuged (10 000 g, 15 min). The pellet containing crude synaptosomes was resuspended in 10 volumes of HEPES-buffered sucrose and centrifuged (10 000 g, 15 min) to yield a washed crude synaptosomal fraction. The synaptosomal fraction was resuspended in 10 mM sucrose, layered onto 1.2 M sucrose and centrifuged (161 000 g, 25 min). The gradient interphase was collected, diluted in HEPES-buffered sucrose and layered on 0.8 M sucrose and centrifuged again (161 000 g, 25 min) to get a pellet containing pure synaptosomes.

2.5. Immunoblotting

WB analysis was performed using Criterion Cell and Criterion Blotter system (BioRad, CA, USA). Equal amounts of the protein were loaded on Criterion 4–20 % pre-cast gel (BioRad), separated by electrophoresis at 200 V for 50 min and electroblotted onto PVDF membrane (Hoefer Scientific Instruments, CA, USA) at 100 V for 60 min. The membranes were blocked with 5% non-fat dried milk powder in TBST (0.05% Tween-20) for 60 min and incubated with primary antibodies and 2.5% non-fat dried milk powder in TBST at RT, ON. The membranes were washed three times for 10 min in TBST and then incubated for 1 h with alkaline–phosphatase linked secondary antibodies and 1.25% non-fat dried milk powder in TBST. The membranes were washed three times for 10 min in TBST. Signals were detected by using ECF substrate (Amersham Biosciences, UK) according to the manufacturers protocol. The membranes were scanned using a fluorescence digital camera detection system (Typhoon scanner).

2.6. Bright field microscopic studies

Free floating vibratome sections (50 µm) from rat brain were treated with 1.0 M ethanolamine-HCL in 0.1 M SPB, pH 7.4 for 30 min. After washing 3 times × 1 min, the sections were incubated in blocking buffer solution (BB) (10% normal goat serum in SPB) for 1 h and then incubated with primary antibody in BB ON/RT. The next day, the sections were rinsed in SPB, 3 times × 5 min and BB for 20 min. The sections were then incubated in biotinylated secondary antibody diluted in BB for 1 h at RT and washed 3 times × 5 min with SPB. The sections were further incubated with streptavidin-biotinylated horseradish peroxidase diluted in BB for one hour and washed in SPB 5 times × 10 min. Finally, the sections were incubated in 0.05% diaminobenzidine (DAB) in SPB for 5 min, before 0.01% H_2O_2 and 0.05% DAB diluted in SPB for 6 min, before final washing in SPB 3 times × 5 min. The sections were mounted on microscope slides with heated glycerol gelatin and cover slides. Images of histological sections were acquired using an automated slide scanner system (Axio Scan Z1, Carl Zeiss Microscopy, Munich, Germany) and Leica Microscope (DM5500B).

2.7. Preparation of hippocampal neuronal cultures

Primary hippocampal cultures containing both neurons and glial cells of 1–4 days old rats were prepared as previously described (Hasegawa et al., 2004). The cultures were maintained in cell medium: Gibcos MEM with the addition of 30 mg/100 ml glutamine, 2.5 mg/100 ml insulin, 5–10 % fetal calf serum, 2 ml/100 ml B-27 and 2–10 µl/100 ml ARA-C in 5% CO_2, in an incubator with 95% air and 5% CO_2 at 37 °C. The cultures were used for experiments after 14–21 days. The cells were fixed in 4.0% FA or 2.5% GA by the following procedure: freshly prepared fixative in 0.1 M SPB was heated to 37 °C before adding it to the culture medium (equal volumes). After 30 min, this mixture was substituted with 0.4% FA or 0.25% GA in 0.1 M SPB ON. The cells were stored in 0.4 F A or 0.25% GA in 0.1 M SPB. The sections were labeled with primary antibody in 2% (v/v) normal calf serum (NCS), 1% (w/v) bovine serum albumin and 0.4% saponin in SPB (ON/RT). The sections were rinsed in SPB, incubated for 30 min with secondary antibodies at RT and rinsed again in SPB. The tissue sections were mounted with fluormont mounting medium (Southern Biotech, Alabama, USA), and images were obtained with an Axioplan 2 equipped with a LSM 5 Pa scanner head (Carl Zeiss, Heidelberg, Germany).

2.8. Perfusion fixation of the rats

For EM studies the rats were deeply anesthetized with Equithesin (0.4 ml/100 g body weight) followed by intracardiac perfusion with 10–15 s flush of 4% Dextran-T70 in SPB (pH 7.4) followed by 2.5% GA (one rat), 4% FA (one rat) or a mixture of 4% FA and 0.1% GA (three rats) in SPB. Each rat was perfused with 500 ml fixative over 15–20 min. The rats were then left ON in a cold room. The next day, the

brains were carefully dissected out and stored in SPB with 0.25% GA, 0.4% FA or a mixture of 0.4% FA/0.01% GA.

2.9. Freeze substitution

Small blocks (0.5–1.0 mm) from the CA1 region of the hippocampus were freeze substituted as follows: The small tissue blocks were cryoprotected in increasing concentrations of glycerol (30 min in 10%, 30 min in 20%, and overnight in 30% at 4 °C) in 0.1 M phosphate buffer and then frozen in a cryofixation unit (Reichert KF80, Vienna, Austria) filled with propane which was cooled down by liquid nitrogen. Afterwards, the tissue was transferred to 1.5% uranyl acetate diluted in anhydrous methanol into the pre-cooled chamber (−90 °C, ON). The tissue pieces were placed in Reichert capsules in a flow-through chamber filled with 1.5% uranyl acetate diluted in anhydrous methanol in a pre-cooled chamber (−90 °C) in a Reichert Automatic Freeze-Substitution unit (AFS) (Leica, Germany). Following 30 h in −90 °C, the temperature was raised with 4 °C increments per hour from −90 °C to −45 °C. The tissue pieces were then rinsed with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Polysciences, Inc., Warrington, PA 18976, Cat#15924). Infiltration in 1:1 and 2:1 in methanol to pure Lowicryl lasted for 2 h each, and 2 h in pure Lowicryl as well as ON in pure Lowicryl. The Reichert capsules were moved to Lowicryl-filled gelatin capsules and then transferred to another container filled with ethanol. The resin/tissue was polymerized with UV-light for 24 h, still at −45 °C. The temperature was increased by 5 °C increments to a final 0 °C where it was polymerized for further 35 h. Ultra-thin sections (90 nm) were cut with a diamond knife (*Diatome* ultra 45°, Diatome, U.S.) on an ultramicrotome (Reichert Ultracut S-2.GA-E-12/92, Leica Microsystems, Germany) and placed on coated (Coat-Quick “G”) nickel grids (Electron Microscopy Sciences, G300-Ni).

2.10. Postembedding immunocytochemistery

Three rats were fixed with a mixture of 4.0% FA and 0.1% GA. Ultrathin sections from the rats were labeled with GA-protein antibodies and standard monoclonal antibodies in a screening experiment. In all three rats, stronger labeling intensity was observed with GA-protein antibodies. The rat with the best ultrastructure was then used for the detailed quantitative analysis (see below). One rat fixed with 2.5% GA and one rat fixed with 4.0% FA were used for immunogold labeling with GA-protein antibodies in order to determine sensitivity of these antibodies for GA-fixed tissue. From each animal, six small (0.5–1.0 mm) blocks from the CA1 region of the hippocampus were freeze substituted. From each animal, one block with the best ultrastructure was selected for quantification. Three ultrathin sections from each block were immunolabeled essentially as described previously (Mathiisen et al., 2006). The sections were incubated in TBST (Tris buffered saline with Tween20) buffer containing 50 mM glycine (10 min) and in TBST containing 2% human serum albumin (HSA) (10 min) to neutralize free aldehyde groups and blocking nonspecific antibody binding sites respectively. The sections were incubated with primary antibody diluted in 2% HSA in TBS with 0.01% Triton X-100) ON at RT. The sections was rinsed and immersed in TBST (10 min) and then incubated in 10 nm goat anti-rabbit IgG colloidal gold-secondary antibody 1:20, in 2% HSA and 1 mg/ml PEG in TBST for 90 min at RT. The sections were then rinsed with distilled water and post-stained with 2% uranyl acetate (90 s) and 0.3% lead citrate (90 s). Uranyl acetate and lead citrate were removed with distilled water, and sections were left to dry completely before examination in the electron microscope. A total of 60 synaptic profiles were used for each antibody for comparing GA-protein antibodies to standard antibodies. A total of 50 synaptic profiles were used for each antibody for comparing sensitivity of GA-protein antibodies for GA and FA fixed tissue. Images with clearly visible pre- and post-synaptic plasma membranes and vesicles membranes were selected for quantitative analysis for each antibody. From each image of a synapse, we examined one region of interest (ROI); either the active zone for SNAP-25 quantification or the presynaptic cytoplasm for VAMP2 quantification.

Pre-incubation experiments: One block with the best ultrastructure from the animals fixed with a combination of 4% FA and 0.1% GA was selected for immunolabeling with anti-GA-SNAP-25 and anti-GA-VAMP2 antibodies after pre-incubation with the same antigen used for immunization of the rabbits. Two sections from the pre-incubation group and two sections from the control group were immunolabeled. Ten synaptic profiles from each section were quantified. A total of 20 profiles were quantified in each group.

SNAP-25 and VAMP2 KO mice: Embryos were killed by decapitation. Left and right hippocampi were dissected and immersed in fixative solution (4.0% FA/0.1% GA SPB). We used immersion fixation of newborn SNAP-25 and VAMP2 KO mice since these animals die immediately after birth. Hippocampi from three KO and three wild-type (WT) mice were embedded with freeze substitution. Two animals/blocks with the best ultrastructure from each group were selected. Two ultrathin sections from each animal/block were immunolabeled. Five synaptic profiles from each section were used for quantification. A total of 20 profiles were quantified in each group.

2.11. EM quantification and statistical analysis

The sections were examined with a Philips Fei Tecnai 12 electron microscope at 60 kV. Electron micrographs were obtained at random from the middle layer of stratum radiatum of the CA1 region of the hippocampus. Specific plasma membrane and cytoplasmic compartments were defined and used for quantifications. SNAP-25 immunolabeling was quantified as number of gold particles/μm over the active zone. VAMP2 immunolabeling was quantified as number of gold particles/μm^2 over the presynaptic cytoplasm. The length of the active zone was defined as equal to the length of the postsynaptic density of the same synapse. Only synaptic profiles with clearly visible synaptic membranes and postsynaptic density were selected for quantitative analysis. The images were quantified with a commercially available image analysis program (analySIS; Soft Imaging Systems, Münster, Germany). Curves were drawn interactively and gold particles were detected semiautomatically. An in-house extension to the analysis software (Soft Imaging Systems) calculated area particle density (number per unit area) over presynaptic cytoplasm for VAMP2 immunogold labeling and linear particle density (number per unit length of curve) over active zone for SNAP-25 immunogold labeling. In the latter case, it measured the distance from each particle-center to the membrane and included only those particles, which were within an operator-defined distance from the curve segment. For general plasma membranes the inclusion distance was symmetric between +/−21 nm. The inclusion distance was defined as the distance between the epitope and the center of the gold particle, corresponding to the radius of the particle (5 nm) and the length of the interposed primary antibody (8 nm) and length of the secondary antibody (8 nm). Data for particles were collected in ASCII files as flat tables and exported to SPSS (SPSS Inc, Chicago, IL, USA) for further statistical and graphical analysis.

3. Results

3.1. Specificity of GA-protein antibodies

We evaluated the specificity of GA-protein antibodies at the electron microscopical level by immunogold labeling of the hippocampus from embryonic KO mice (Fig. 1). Quantification of SNAP-25 gold particles over the active zone of presynaptic terminals (Fig. 1A) revealed only 18% labeling density in the KO mice (Fig. 1B4) compared to the WT mice (Fig. 1B3) (n = 20 in both groups, WT: 10.1 ± 1.9, KO: 1.8 ± 0.75, $p < 0.001$, Mann-Whitney *U* test). Quantitative analysis of VAMP2 labeling over the presynaptic cytoplasm (Fig. 1C) showed

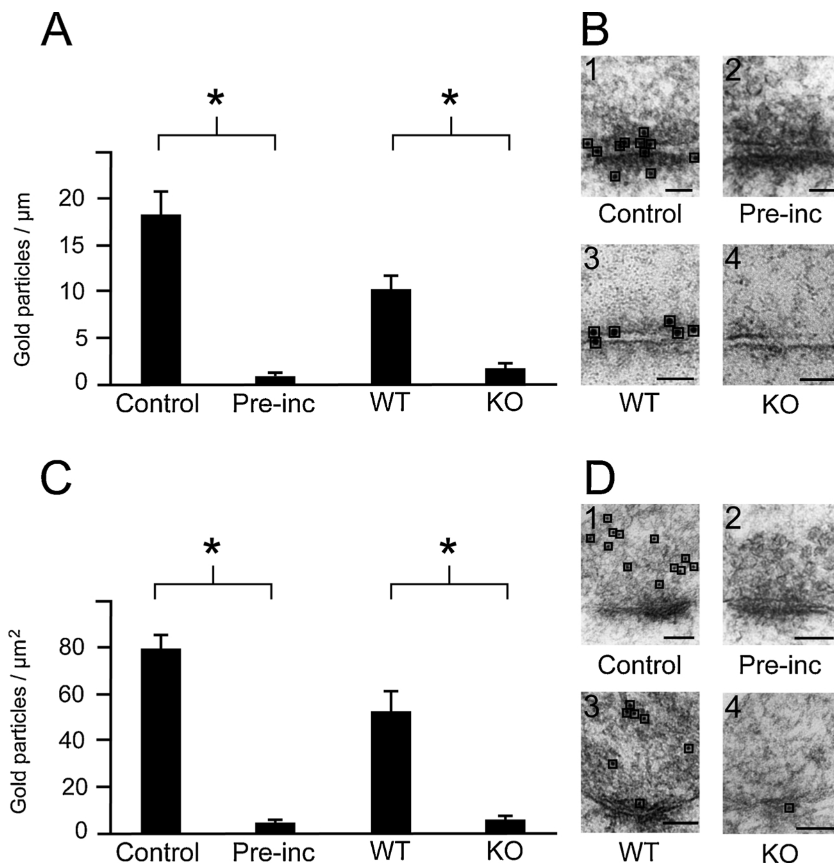

Fig. 1. Specificity of anti-GA-SNAP-25 and anti-GA-VAMP2 antibodies.
(A) Quantitative analysis of immunogold labeling with anti-GA-SNAP-25 antibody at the active zone of hippocampal synapses, preincubated with SNAP-25 protein and synapses from SNAP-25 KO mice. (B) Electron micrographs displaying corresponding SNAP-25 immunogold labeling. Gold particles are highlighted with squares. (B1) Standard labeling. (B2) Labeling with antibody pre-incubated with SNAP-25 antigen. (B3) Standard labeling of WT mice. (B4) Labeling of SNAP-25 KO tissue. (C) Quantitative analysis of immunogold labeling with anti-GA-VAMP2 antibody of presynaptic cytoplasm at hippocampal synapses, preincubated with VAMP2 protein and synapses from VAMP2 KO mice. (D) Electron micrographs displaying VAMP2 immunogold labeling. Gold particles are highlighted with squares. (D1) Standard labeling. (D2) Labeling with antibody pre-incubated with VAMP2 antigen. (D3) Standard labeling of WT mice. (D4) Labeling of VAMP2 KO tissue. Embryonic (E18) WT and KO mice were used for immunogold labeling. Pre-incubation experiments were performed in adult rats. Scale bars (B) 50 nm. (D) 100 nm.

only 13% labeling intensity in the KO animals (Fig. 1D4) compared to the WT mice (Fig. 1D3) (n = 20 in both groups, WT: 52.5 ± 9.1, KO: 6.7 ± 2.0, $p < 0.001$, Mann-Whitney *U* test). We further performed immunogold labeling of sections from adult rat hippocampus with GA-protein antibodies after pre-incubation with the same antigen used for immunization of the rabbits (Fig. 1B2, D2). The analysis showed only 4% labeling density in the pre-incubation group for SNAP-25 (Fig. 1A) compared to control (n = 20 in both groups, ctrl: 18.2 ± 2.5, pre-inc: 0.8 ± 0.5, $p < 0.001$, Mann-Whitney *U* test) and 6% for VAMP2 (Fig. 1C) (n = 20 in both groups, ctrl: 79.4 ± 6.2, pre-inc: 4.7 ± 1.7, $p < 0.001$, Mann-Whitney *U* test).

3.2. Antibody sensitivity for fixed and non-fixed protein

In order to determine sensitivity of GA-protein antibodies compared to that of commercially available standard antibodies for fixed protein, we applied them to brain homogenate and synaptosomes on PVDF membranes after fixation with a combination of GA and FA (Fig. 2). Staining of brain homogenate and synaptosome fraction with anti-GA-SNAP-25 and anti-GA-VAMP2 detected bands at respectively 25 and 18 kDa, the predicted molecular weight of these proteins. Anti-GA-SNAP-25 antibody showed stronger bands on the fixed membrane compared to the non-fixed membrane (Fig. 2A). Conversely, standard poly- and monoclonal SNAP-25 antibodies showed stronger affinity for proteins on the normal, unfixed membrane (Fig. 2B, C). Similar results were observed with the anti-GA-VAMP2 antibody; immunostaining revealed stronger band intensities on the fixed membrane (Fig. 2D), while standard VAMP2 antibodies binds stronger to the non-fixed membrane (Fig. 2E, F).

3.3. Immunofluorescence labeling of neuronal cultures

To further verify the quality of our antibodies, we performed immunofluorescence labeling of dissociated hippocampal neuronal cultures. GA-protein antibodies and standard antibodies were applied to GA-fixed and FA-fixed neurons (Fig. 3). Staining with anti-GA-SNAP-25 showed distinct synaptic and dendritic profiles with limited background (Fig. 3A and D). Immunolabeling with standard polyclonal and monoclonal SNAP-25 antibodies displayed blurred synapses and weak dendritic profiles (Fig. 3B, C, E, F). Staining of neuronal cultures with anti-GA-VAMP2 showed clear, round and sharp synaptic profiles. No background labeling was observed (Fig. 3G, J). Staining with standard VAMP2 polyclonal antibody (Fig. 3H, I, K, L) showed more background and round blurry boutons with different sizes compared to labeling with in house antibody.

3.4. Quantitative postembedding electron microscopy

To evaluate performance of the antibodies at the ultrastructural level, we performed postembedding immunogold labeling of ultrathin sections from the CA1 region of the hippocampus (Fig. 4). SNAP-25 is a t-SNARE localized to the plasma membrane and found in high concentrations at sites for exocytosis of vesicles, like the presynaptic active zone. Labeling with anti-GA-SNAP-25 was membrane associated as expected. Many gold particles were located at the active zone (Fig. 4A). Labeling with standard poly- and monoclonal SNAP-25 antibodies showed low gold particle densities at the active zone (Fig. 4B, C). VAMP2 is a v-SNARE localized on synaptic vesicles. Labeling with anti-GA-VAMP2 was consistent with the expected ultrastructural localization of the protein. Strong labeling intensity of vesicles in the presynaptic cytoplasm was observed (Fig. 4D). Standard VAMP2 polyclonal and monoclonal antibodies also displayed the expected pattern, however, the labeling intensity was low compared to GA-VAMP2 antibody (Fig. 4E, F).

We then performed quantitative analyses of the immunogold labeling described above. SNAP-25 immunolabeling was quantified over

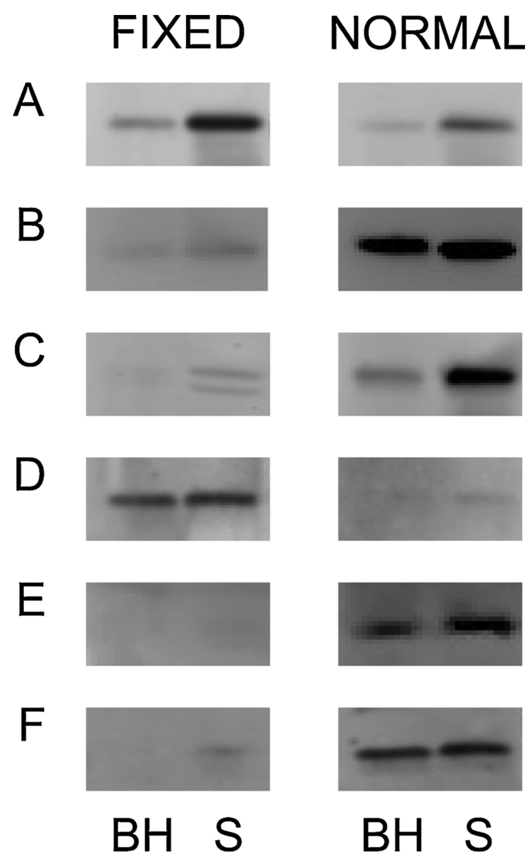

Fig. 2. Comparison of anti-GA-SNAP-25 and anti-GA-VAMP2 antibodies with corresponding standard antibodies on fixed and unfixed WB membranes.
Western blots of rat brain homogenate (BH) and synaptosomes (S) labeled with anti-GA-SNAP-25 and anti-GA-VAMP2 antibodies and corresponding standard antibodies against SNAP-25 and VAMP2. Antibodies were applied on blotted membranes fixed with a combination of 4.0% FA + 0.1% GA or on normal non-fixed membranes. (A) Anti-GA-SNAP-25. (B) Standard polyclonal anti-SNAP-25. (C) Standard monoclonal anti-SNAP-25. (D) Anti-GA-VAMP2. (E) Standard polyclonal anti-VAMP2. (F) Standard monoclonal anti-VAMP2.

the active zone as gold particles/μm membrane length. Mean gold particle density with anti-GA-SNAP-25 was 35.7 ± 2.1 (Fig. 4G). The corresponding value for standard polyclonal and monoclonal antibodies against SNAP-25 was 4.1 ± 0.8 and 4.0 ± 0.8 (Fig. 4G). Statistical calculation revealed significant differences between GA-SNAP-25 antibody and both of the standard antibodies (n = 60 in each group, $p <$ 0.001, Mann-Whitney *U* test). Analysis did not show significant differences between the standard poly and monoclonal antibodies, (n = 60 in both groups, $p =$ 0,998, Mann-Whitney *U* test). Mean gold particles/μm^2 over presynaptic cytoplasm with the anti-GA-VAMP2 was 248.3 ± 9.1 (Fig. 4H). The mean numbers of gold particles/μm^2 with the standard polyclonal and monoclonal antibodies against VAMP2 were respectively 29.2 ± 2.2 and 39.6 ± 3.0 (Fig. 4H). Statistical analysis revealed significant differences in labeling intensity between GA-VAMP2 antibody and the both the standard antibodies (n = 60 in each group, $p <$ 0.001, Mann-Whitney *U* test). No significant change was detected in labeling density between the standard poly and monoclonal VAMP2 antibodies (n = 60 in both groups, $p =$ 0.433, Mann-Whitney *U* test).

3.5. Sensitivity of GA-protein antibodies for glutaraldehyde fixed tissue

Our antibodies were raised against GA-fixed antigen. To clarify whether these GA-protein antibodies have higher affinities for protein fixed with GA compared to FA fixed proteins, the GA-protein antibodies were applied to ultrathin brain sections fixed with GA and ultrathin brain sections fixed with FA (Fig. 5). Immunogold labeling with anti-GA-SNAP-25 and anti-GA-VAMP2 showed significantly higher labeling density, respectively, of the active zone (Fig. 5A) and the presynaptic cytoplasm (Fig. 5D) in GA compared to FA fixed brains (Fig. 5B, E). Quantification of SNAP-25 immunogold labeling over the active zone revealed 18.9 ± 1.2 gold particles in GA and 5.1 ± 0,8 in FA fixed synapses (Fig. 5C) (n = 50 in both groups, $p <$ 0.001, Mann-Whitney *U* test). Mean number of gold particles/μm^2 over the presynaptic cytoplasm with anti-GA-VAMP2 was 184.9 ± 6.7 in GA and 43.9 ± 3.3 in FA fixed tissue (Fig. 5F) (n = 50 in both groups, $p <$ 0.001, Mann-Whitney *U* test).

Affinity of our GA-protein antibodies for GA-fixed epitopes was additionally evaluated by DAB peroxidase labeling of LM sections fixed with GA and LM sections fixed with FA (Fig. 6). Staining intensity was clearly stronger in the GA-fixed brain slices with both GA-SNAP-25 and GA-VAMP2 antibody (Fig. 6A, C). Labeling was characteristically punctate in the synaptic areas of hippocampal CA1 region in the GA group, while labeling was almost absent or very weak in the same area of FA-fixed brain (Fig. 6B, D). Syntaxin1 is a t-SNARE with a similar localization pattern as SNAP-25 in synapses. DAB peroxidase labeling with commercially available standard anti-syntaxin1 raised against non-fixed antigen was used as control. Staining of LM sections with anti-syntaxin1 displayed equally strong labeling intensity of GA- and FA-fixed brain sections (Fig. 6E, F).

4. Discussion

In addition to conventional problems like antibody specificity, tissue treatment procedures necessary for EM visualization almost without exception mask or denature the protein antigens in a way to hinder antibody binding (Mathiisen et al., 2006). One way of overcoming many of these problems, is to perform pre-embedding labeling, prior to many of the denaturing procedures. However, pre-embedding techniques are not optimal for quantitative analysis due to local differences in penetrability of the tissue. Among the denaturing or masking procedures are: fixation with GA, contrast staining with osmium, uranyl acetate and lead citrate, dehydration, and embedding in resin. In addition, many resins require elevated temperatures to cure. Thus, in order to minimize denaturing of proteins during the embedding procedure, we used a well established, freeze substitution procedure (Mathiisen et al., 2006) where tissue is dehydrated and embedded under very low temperatures, also allowing a not complete dehydration, thus minimizing the usual denaturing effects. In addition, this material is not post-fixed in osmium, and the concentration of GA is kept to a minimum (0.1–0.25%, compared to, e.g., 2.5–4.0 % in conventional EM techniques). However, the ultrastructure of the tissue is poor compared to conventional EM. Still, even with these procedures, postembedding immunogold labeling is a challenge. The relatively weak fixation with GA still denatures the proteins sufficiently to reduce antibody binding efficiency. For structural protein antigens present in high concentrations in the tissue, this may not always pose a practical problem. However, for regulatory proteins, e.g. SNARE proteins or neurotransmitter receptors, this may still present a significant challenge. With such proteins, present in the membrane or cytoplasm in comparatively low concentrations, only few commercially available antibodies against peptides provide a quantifiable signal with these EM procedures.

The specificity of our antibodies were evaluated at ultrastructural level by immunogold labeling of brain sections from the KO mice and immunogold labeling after pre-incubation with the same antigen used for immunization of the rabbits. The results clearly demonstrated that denaturation of antigen prior to immunization does not reduce the specificity of the antibodies (Fig. 1).

Others have tried a similar approach previously (Harrach and Robenek, 1990). They raised polyclonal antibodies against

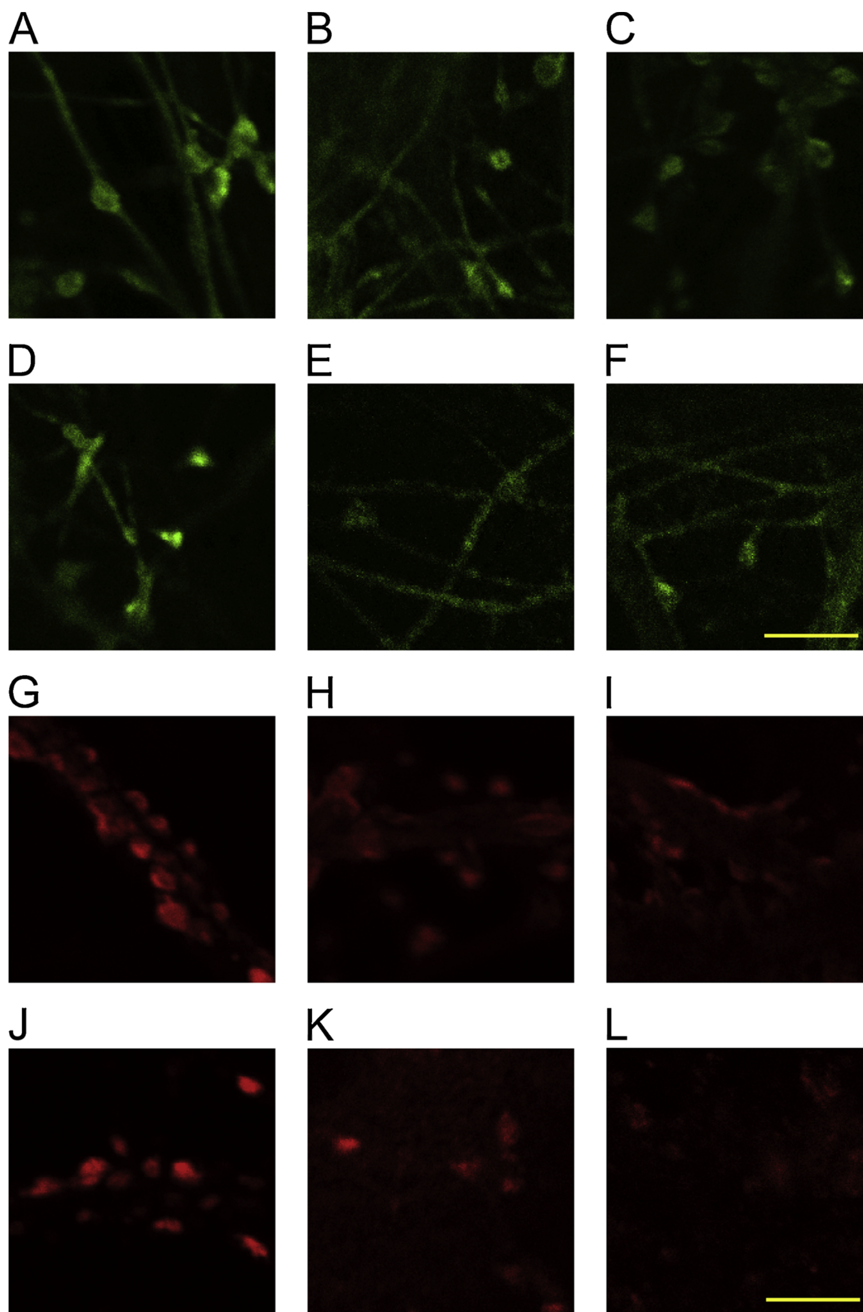

Fig. 3. Immunofluorescence staining of aldehyde-fixed dissociated hippocampal cultures with anti-GA-SNAP-25 and anti-GA-VAMP2, compared to staining with corresponding standard antibodies.
Confocal images of dissociated hippocampal neuronal cultures immunostained with anti-GA-SNAP-25, anti-GA-VAMP2, or corresponding standard antibodies against the same proteins. The antibodies were applied to neuronal cultures fixed with 2.5% GA and neuronal cultures fixed with 4.0% FA. (A) GA fixed neuronal culture stained with anti-GA-SNAP-25. (B) GA fixed neuronal culture stained with standard polyclonal anti-SNAP-25. (C) GA fixed neuronal culture stained with standard monoclonal anti-SNAP-25. (D) FA fixed neuronal culture stained with anti-GA-SNAP-25. (E) FA fixed neuronal culture stained with standard polyclonal anti-SNAP-25. (F) FA fixed neuronal culture stained with standard monoclonal anti-SNAP-25. (G) GA fixed neuronal culture stained with anti-GA-VAMP2. (H) GA fixed neuronal culture stained with standard polyclonal anti-VAMP2. (I) GA fixed neuronal culture stained with standard monoclonal anti-VAMP2. (J) FA fixed neuronal culture stained with anti-GA-VAMP2. (K) FA fixed neuronal culture stained with standard polyclonal anti-VAMP2. (I) FA fixed neuronal culture stained with standard monoclonal anti-VAMP2. Scale bars: 5 µm.

apolipoprotein pre-fixed with FA. The antibodies reacted specifically and recognized fixed as well as unfixed apolipoprotein in ELISA and WB analysis. The efficacy of the antibodies was further tested at the electron microscopical level showing specific labeling localized to the cisternae of the

rough endoplasmic reticulum, the Golgi complex, and in vesicles associated with the Golgi region. Furthermore, Harrach and colleagues were able to localize apolipoprotein in foam cells of the human aortic plaque by postembedding immunocytochemistry. In control experiments carried out by using antibodies pre-adsorbed with the antigen, no labeling was observed. Riederer also generated antibodies against antigen pre-fixed with FA for use in immunohistochemical procedures (Riederer, 1993). The large subunit of neurofilaments was exposed to FA and used for immunization of rabbit. In aldehyde fixed cerebellum, the antibody strongly stained axons. In contrast, in alcohol-fixed cryostat sections the immunocytochemical detection was substantially reduced. Danbolt and colleagues (Danbolt et al., 1992) successfully raised antibodies against antigen treated with a mixture of FA and GA for use in immunohistocytochemical applications. We (Bock et al., 1997) and others (Fujiwara and Masuyama, 1995; Mera et al., 2008) have also used GA as cross-linker to conjugate proteins and produce epitope-specific antibodies. Although there are several studies reporting the potential of antibodies made against fixed proteins, no other studies have so far systematically evaluated the quality of antibodies raised against antigen pre-fixed with GA for use in EM.

In the present study we show that antibodies raised against fixed antigen in fact more readily binds to fixed, denatured, protein compared to native, unfixed protein, while reduced antigenicity of fixed proteins were observed for the commercial antibodies. The concentration of GA-protein antibodies was kept low to highlight the differences, at higher concentration these antibodies displayed strong immunoreactivity also of untreated antigen. Thus, our antibodies were shown to interact satisfactorily with both aldehyde treated antigen and normal antigen in a biochemical application.

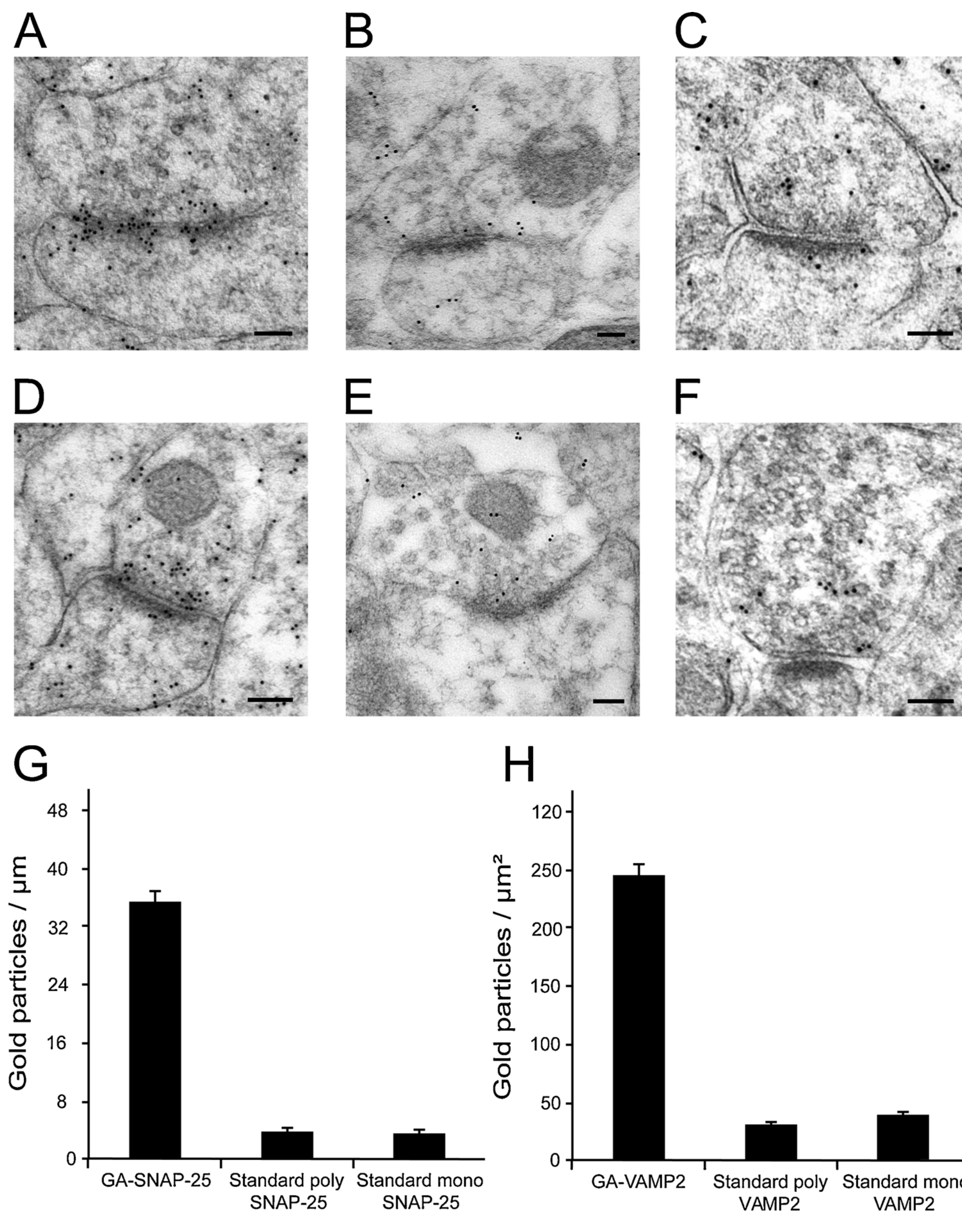

Fig. 4. Quantitative EM of synapses in the hippocampus labeled with anti-GA-SNAP-25, anti-GA-VAMP2, or corresponding standard antibodies.
Electron micrographs showing SNAP-25 and VAMP2 immunogold labeling of asymmetric synapses from the CA1 region of the hippocampus fixed with a combination of 4.0% FA + 0.1% GA. (A) Anti-GA-SNAP-25 labeling. (B) Standard polyclonal anti-SNAP-25 labeling. (C) Standard monoclonal anti-SNAP-25 labeling. (D) Anti-GA-VAMP2 labeling. (E) Standard polyclonal anti-VAMP2 labeling. (F) Standard monoclonal anti-VAMP2 labeling. (G) Quantitative analysis of SNAP-25 immunogold labeling. (H) Quantitative analysis of VAMP2 immunogold labeling. Scale bars: 100 nm.

However, conditions are different in immunocytochemical staining of intact cells. Fixation of tissue with GA decreases antibody penetration of antibodies (Pow, 1997). Moreover, GA fixation creates autofluorescent compounds (Rost and Ewen, 1971) and has shown to increase background immunofluorescence due to non-specific binding of antibodies to unintended proteins (Mundegar et al., 2008). In our study, we did not observe autofluorescence or decreased tissue penetration of the antibodies, indicating advantage of GA-proteins antibodies for immunofluorescence assays. Furthermore, significantly stronger labeling intensity of GA fixed brains compared to FA treated sections with our antibodies confirmed that immunoreactivity significantly increases by selecting the same fixative for fixation of the tissue as for modifying antigen before immunization.

The main purpose of making antibodies against GA treated antigens was to improve immunolabeling at the ultrasctructural level. We took advantage of GA-SNAP-25 and GA-VAMP2 antibodies in our recent publications (Hussain and Davanger, 2015; Hussain et al., 2018). In these studies we showed novel electron microscopical data of SNAP-25 and VAMP2 expression in postsynaptic spines from the CA1 region of the hippocampus. We believe that these antibodies have uncovered localization of SNARE proteins in the brain that has not been demonstrated previously. In the present work, our antibodies produced strong labeling of synaptic terminals. Though also standard antibodies showed labeling of VAMP2 and SNAP-25 positive structures, the labeling intensity was not satisfying despite high concentrations of the antibodies. Thus, GA-protein antibodies were shown to be superior to corresponding standard commercial antibodies. Our results highlight the experimental potential of raising antibodies against GA-treated antigen to improve sensitivity of the antibodies for postembedding immunogold EM.

Declaration of interest

The University of Oslo supported this work. The authors declare that they have no competing interests.

Ethical standards agreement

I have read and have abided by the statement of ethical standards for manuscripts submitted to the Journal of Neuroscience Methods.

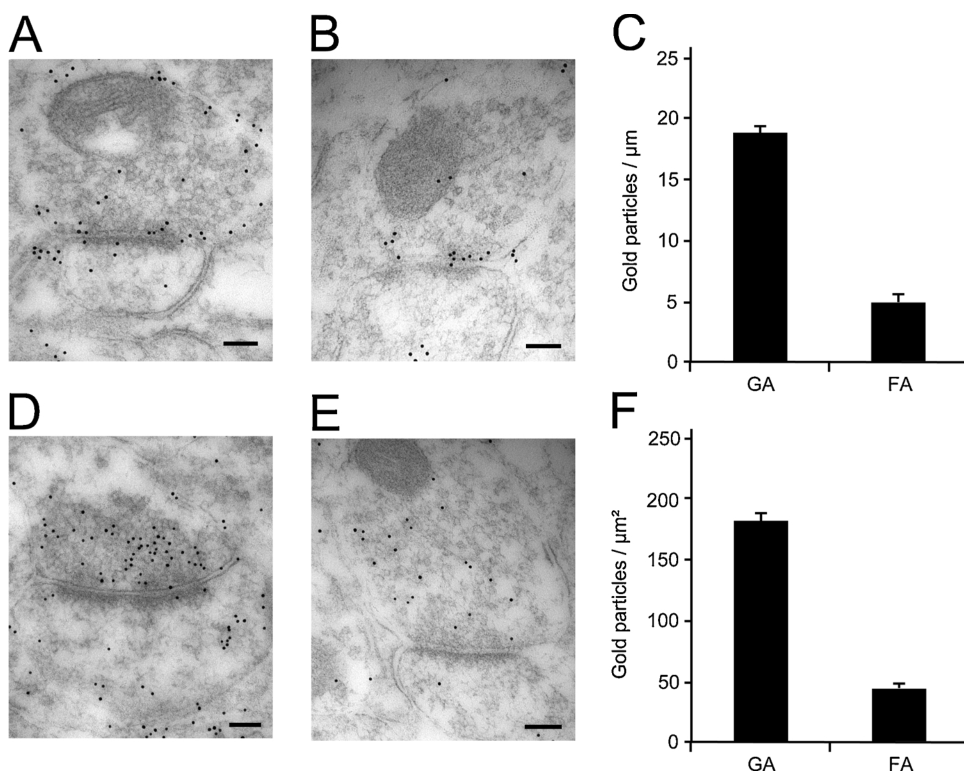

Fig. 5. Sensitivity of anti-GA-SNAP-25 and anti-GA-VAMP2 in tissues fixed with different aldehydes at the electron microscopical level. Anti-GA-SNAP-25 and anti-GA-VAMP2 antibodies were applied to ultrathin brain sections fixed with 2.5% GA and ultrathin brain sections fixed with 4.0% FA. (A) GA fixed brain sections labeled with anti-GA-SNAP-25. (B) FA fixed brain sections labeled with anti-GA-SNAP-25. (C) Quantitative analysis of SNAP-25 immunogold labeling. (D) GA fixed brain sections labeled with anti-GA-VAMP2. (E) FA fixed brain sections labeled with anti-GA-VAMP2. (F) Quantitative analysis of VAMP2 immunogold labeling. Scale bars: 100 nm.

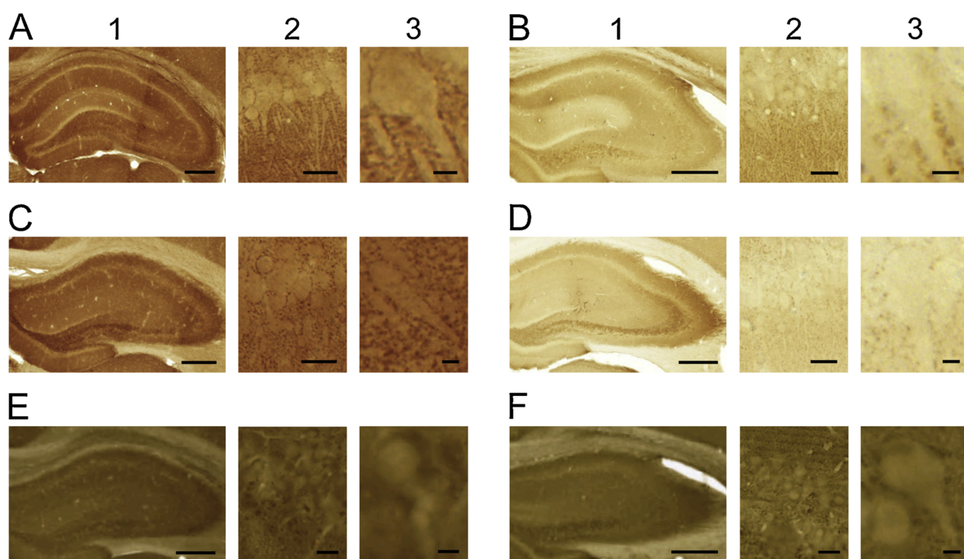

Fig. 6. Light micrographs of GA and FA fixed rat brain sections stained with GA-protein antibodies and standard syntaxin1 antibody. Immunoperoxidase staining of vibratome sections with anti-GA-SNAP-25, anti-GA-VAMP2 and standard anti-syntaxin1. For each of the antibodies, brain tissue fixed with 2.5% GA and brain tissue fixed with 4.0% FA were immunostained. (A) GA fixed brain sections labeled with anti-GA-SNAP-25. (B) FA fixed brain sections labeled with anti-GA-SNAP-25. (C) GA fixed brain sections labeled with anti-GA-VAMP2. (D) FA fixed brain sections labeled with anti-GA-VAMP2. (E) GA fixed brain sections labeled with standard anti-syntaxin1. (F) FA fixed brain sections labeled with standard anti-syntaxin1. Scale bars: (A1-F1) 500 µm. (A2-F2) 25 µm. (A3, B3, D3, E3, F3) 5 µm. (C3) 10 µm.

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