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Native Presynaptic Metabotropic Glutamate Receptor 4 (mGluR4) Interacts with Exocytosis Proteins in Rat Cerebellum*[S]

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Background: mGluR4 negatively modulates neurotransmission at glutamatergic synapses.
Results: Using proteomic approaches, we show that native mGluR4 interacts with exocytosis proteins.
Conclusion: Native mGluR4 could inhibit glutamate release via interactions with exocytosis proteins.
Significance: Because mGluR4 plays key roles in regulation of neurotransmission and is considered as a promising target for treatment of various brain diseases, the identification of its mechanisms of action is fundamental.

The eight pre- or/and post-synaptic metabotropic glutamatergic receptors (mGluRs) modulate rapid excitatory transmission sustained by ionotropic receptors. They are classified in three families according to their percentage of sequence identity and their pharmacological properties. mGluR4 belongs to group III and is mainly localized presynaptically. Activation of group III mGluRs leads to depression of excitatory transmission, a process that is exclusively provided by mGluR4 at parallel fiber-Purkinje cell synapse in rodent cerebellum. This function relies at least partly on an inhibition of presynaptic calcium influx, which controls glutamate release. To improve the understanding of molecular mechanisms of the mGluR4 depressant effect, we decided to identify the proteins interacting with this receptor. Immunoprecipitations using anti-mGluR4 antibodies were performed with cerebellar extracts. 183 putative partners that co-immunoprecipitated with anti-mGluR4 antibodies were identified and classified according to their cellular functions. It appears that native mGluR4 interacts with several exocytosis proteins such as Munc18-1, synapsins, and syntaxin. In addition, native mGluR4 was retained on a Sepharose column covalently grafted with recombinant Munc18-1, and immunohistochemistry experiments showed that Munc18-1 and mGluR4 colocalized at plasma membrane in HEK293 cells, observations in favor of an interaction between the two proteins. Finally, affinity chromatography experiments using peptides corresponding to the cytoplasmic domains of mGluR4 confirmed the interaction observed between mGluR4 and a selection of exocytosis proteins, including Munc18-1. These results could give indications to explain how mGluR4 can modulate glutamate release at parallel fiber-Purkinje cell synapses in the cerebellum in addition to the inhibition of presynaptic calcium influx.

Glutamate mediates fast excitatory synaptic transmission by activating ionotropic receptors in the central nervous system. This neurotransmitter can also activate G protein-coupled receptors such as metabotropic glutamate receptors so called mGluRs.[3] These receptors display differential expression on pre- and postsynaptic membranes and modulate the fast excitatory glutamatergic neurotransmission. This family is divided into three groups (I, II, III) according to their sequence identities, pharmacological profile, and signal transduction mechanisms. Group III mGluRs have been described to be negatively coupled to adenylate cyclase (1) and have been shown to inhibit glutamate release (2, 3). These receptors are activated by released glutamate in the synaptic cleft and act as autoreceptors to inhibit excitatory synaptic transmission. They are also involved in the induction of two forms of short-term synaptic plasticities: the paired-pulse facilitation and the post-tetanic potentiation (4, 5). The mGluR4 subtype, which belongs to the presynaptic group III mGluRs, is mainly expressed in the cerebellar cortex (6). The most compelling demonstration for a physiological function of presynaptic mGluR4 is found at the glutamatergic synapses between parallel fibers and Purkinje cells in the rodent cerebellar cortex. At these synapses, pharmacological activation of these receptors acutely depresses excitatory transmission and inhibits presynaptic calcium influx (7, 8). In the rodent cerebellum, depression of parallel fiber-Purkinje cell excitatory transmission is exclusively due to mGluR4 (8).

Little is known about the mechanisms of action of mGluR4 and the presynaptic targeting of these receptors. Most of the studies conducted to identify mGluR4-interacting proteins were performed in GST pulldown assays or in heterologous

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[S] This article contains supplemental Table 1.
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[3] The abbreviations used are: mGluR, metabotropic glutamatergic receptor; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP-25, synaptosomal-associated protein 25; CB, Cascade Blue; HBSS, Hanks' buffered salt solution; IP, immunoprecipitation; TX-100, Triton X-100; PICK1, protein interacting with c-kinase 1; SV2A, synaptic vesicle glycoprotein 2A.

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systems. mGluR4 has been shown to interact with microtubule-associated protein or with proteins linked to cytoskeleton (β-spectrin, myosin) or involved in endocytosis (clathrin and adaptor complex proteins) (9). The Ca^{2+} sensor calmodulin has also been reported to interact with the C-terminal tail of mGluR4 (10) as well as syntenin, glutamate receptor interacting protein (11), and calcium-binding protein 1 (12). Finally, Munc18-1 was also recently identified as a binding partner for mGluR4 by co-immunoprecipitation and GST pulldown experiments (13). This protein has been suggested to assist in the formation of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex syntaxin/synaptosomal-associated protein 25 (SNAP-25)/synaptobrevin during neurotransmitter vesicles exocytosis (14) via a Ca^{2+}/ calmodulin-dependent pathway.

In an attempt to identify new molecular partners involved in the negative regulation of neurotransmission induced by native mGluR4 in the rat cerebellum, we decided to carry out a proteomic study. Several papers reported proteomic approaches for the identification of proteins interacting with G-protein-coupled receptor such as 5-hydroxytryptamine type 2 receptor (15) and mGluR5 (16). These works led to the discovery of direct but also indirect interactions, all partners included in the term "receptosome." To study the receptosome of mGluR4, we used a proteomic approach consisting of co-immunoprecipitation of the native receptor and identification of the protein partners by mass spectrometry analysis. In this study we demonstrated that mGluR4 was able to interact with numerous exocytosis proteins including Munc18-1 and SNAREs proteins such as syntaxin and SNAP-25. The interaction of Munc18-1 with native mGluR4 from rat cerebellum was confirmed using chromatography on a Sepharose column grafted with recombinant Munc18-1. Interaction domains of mGluR4 with several exocytosis proteins were further studied by affinity chromatography experiments using peptides corresponding to the intracellular parts of mGluR4. These interactions could be involved in the molecular mechanisms underlying the depressant presynaptic action of this activated receptor in the cerebellar cortex through the control of glutamate release.

EXPERIMENTAL PROCEDURES

Material—Two anti-mGluR4 antibodies were used, one for co-immunoprecipitation (ab51–3100, Invitrogen) and another for receptor detection in Western blot experiments (AB15097, Millipore, Bedford, MA). Anti-Cascade Blue antibody (CB, Molecular Probes, Eugene, OR) was used as a negative control in all co-immunoprecipitation experiments. Other antibodies used in Western blotting experiments were purchased from Abcam (Cambridge, UK): Munc18-1 (ab3451), syntaxin (ab39289), synapsin I (ab8), synapsin II (ab13258), SNAP-25 (ab5666). Antibodies used in co-localization experiments were: mouse anti-Munc18-1 (Transduction Laboratories, Lexington, KY), chicken anti-HA (AvesLAB, Tigard, OR), Alexa488 donkey anti-chicken (Interchim, Montluçon, France), and Alexa546 goat anti-mouse (Molecular Probes). Detergents (Triton X-100 and Digitonin) were purchased from Sigma, antiprotease mixtures were from Roche Applied Science, and modified trypsin was from Promega (Madison, WI).

Glutamate Assay—Glutamate assay was performed on total protein lysate of rat cerebellum according to the instructions of the manufacturer (EnzyChrom™ Glutamate Assay kit, BioAssay Systems, Hayward, CA).

Immunoprecipitation—Animal care and all experiments procedures adhered to governmental guidelines. Animals were stunned and then decapitated. Proteins were manually extracted using a potter from cerebellum of 30–37-day-old male Sprague-Dawley rats with lysis buffer in a ratio of 0.25 ml/10 mg of tissue. The buffer contained 150 mM NaCl, 25 mM HEPES-NaOH, pH 8.0, 2% digitonin, 0.5 mM EDTA, 0.25 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 0.01 mg/ml E-64, 0.05 mg/ml antipaïn, and 1 mM Na_3VO_4. The homogenate was incubated on a rotary shaker during 1 h at 8 °C then centrifuged at 14,000 × *g* for 20 min at 4 °C. Supernatant (1 ml) was used for immunoprecipitation. Protein concentration was determined by the Bradford method. Anti-mGluR4 and anti-Cascade Blue antibodies were cross-linked to protein A-Sepharose magnetic beads (Ademtech, Pessac, France) according to instructions of the manufacturer. The supernatant (3 mg of proteins) was incubated with 9 μg of anti-mGluR4 or anti-CB overnight at 4 °C. After several washing steps with 150 mM NaCl, 25 mM HEPES-NaOH, pH 8.0, buffer, proteins were eluted with 30 μl of 50 mM glycine, pH 2.5, loaded on 10% acrylamide SDS-PAGE without heating, and then revealed with silver nitrate staining or Western blot.

Protein Identification by Mass Spectrometry—After one-dimensional electrophoresis, bands of interest were excised, then treated and digested using the automated system Digest Pro 96 (Intavis AG, Bremen, Germany). Bands were first destained by two washing steps with a freshly prepared solution containing 15 mM $K_3[Fe(CN)_6]$ and 50 mM $Na_2S_2O_3$. Then, proteins were reduced and alkylated by successive incubations with 10 mM DTT in 50 mM NH_4HCO_3 for 30 min at 57 °C and 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 20 min at room temperature. In-gel digestion was then performed with modified trypsin (Promega), and peptides were extracted with formic acid-CH_3CN (1:60 ratio). Peptide mixture was submitted to nano-LC-ion trap (IT)-MS/MS using an Agilent 1200 nanoflow LC system coupled to a 6330 Ion Trap equipped with the Chip Cube orthogonal ionization system (Agilent Technologies, Santa Clara, CA) as described in Marchand *et al.* (17). For protein identification, a MASCOT MS/MS Ions search was used, and searches were performed against the NCBI data base (release20100116; 10.343.571 sequences) with taxonomic specification to *Rattus norvegicus*. Mass accuracy tolerance was set to 100 ppm on the parent ion mass and 0.5 Da in MS/MS mode. One missed cleavage per peptide was allowed, and some modifications were taken into account: carbamido-methylation for cysteines as fixed modification and methionine oxidation as variable modifications. Proteins were considered with confidence if at least two unique peptides were identified with a score $\geq$34 and if the p value was $<$0.03. The Mowse score for the protein is calculated as $-10 \times \log(p)$.

Protein Munc18-1 Overexpression and Purification—Coding sequence of rat Munc18-1 was cloned under the T7 promotor control into PIVEX2.4d plasmid, allowing the addition of a six-histidine tag at the N terminus of the protein. We used a syn-

thetic gene optimized for heterologous expression and supplied by MWG. BL21 (λDE3/pDIA17) was used as expression strain. Cells were grown under aerobic conditions at 37 °C in Luria-Bertani medium in the presence of 0.1 mg/ml ampicillin, and protein expression was induced for 2 h with 0.5 mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested by centrifugation and broken in French press at 4 °C in lysis phosphate buffer, pH 7.5, containing 2 mM $MgSO_4$, 5% sucrose, 100 mM NaCl, 0.01 mg/ml DNase, and a mixture of anti-proteases (Complete Protease Inhibitor Mixture Tablet, Roche Applied Science). After cell debris and inclusion bodies had been pelleted, cytoplasmic and membrane proteins were separated by ultracentrifugation (90,000 × *g*, 1 h, 4 °C). The Munc18-1 recombinant protein found in the supernatant was affinity-purified on a Ni^{2+}-IDA 1000 column (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. The protein was eluted from the affinity column with 250 mM imidazole, then submitted to dialysis against buffer, pH 8.3, containing 100 mM $NaHCO_3$ and 500 mM NaCl. The concentration of purified Munc18-1 was determined spectrophotometrically (ϵ_{280} = 61,770 M^{-1} cm^{-1}).

Cell Culture, Transfection, and Immunoassay—HEK293TSA cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate (Invitrogen), and a mix of penicillin/streptomycin. 4×10^4 cells were plated in a 12-well plate containing 18-mm coverslips and coated with polyornithine. After 1 day, cells were co-transfected with PRK5-HAmGluR4 and pUC57-Munc18-1 using the Jetprime transfection reagent (PolyPlus). After 1 day, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100, and blocked with phosphate-buffered saline (PBS) complemented with 5% horse serum. Munc18-1 and mGluR4 proteins were incubated, respectively, with mouse anti-Munc18-1 (1/300) and chicken anti-HA (1/300) antibodies for 1 h. After washes with PBS, secondary antibodies Alexa488 donkey anti-chicken and Alexa546 goat anti-mouse (1/500) were then incubated. Finally, cells were washed, mounted in Vectashield, and imaged using a Zeiss Axioimager microscope.

Co-capping Assay—To cap surface mGluR4, cells were incubated with cold anti-HA antibody (1/50, in Hanks' buffered salt solution (HBSS), 1% horse serum) for 30 min on ice. Then cells were washed in ice-cold HBSS and labeled with Alexa488 secondary antibody (1/100, in HBSS 1% horse serum). Cells were incubated for 1 h at 37 °C and then fixed with 4% paraformaldehyde for 15 min. To label Munc18-1, cells were permeabilized with 0.2% Triton X-100 and incubated with anti-Munc18-1 antibody (1/50, in HBSS, 1% horse serum) for 30 min at room temperature and washed with PBS. Munc18-1 was detected by incubating cells with Alexa546 secondary antibodies (1/100, in HBSS, 1% horse serum) for 30 min at room temperature. Finally, cells were washed, mounted in Vectashield, and imaged using a ZEISS LSM510 confocal microscope.

Colocalization Quantification—Colocalization was determined using ImageJ software (National Institute of Health) and the Coloc_2 plugin. Basically, the plugin performed a pixel intensity spatial correlation analysis. We used the Mander's coefficient (*r*) (0 is no colocalization, 1 means 100% colocalization) to evaluate the signal proportion in one channel that colocalizes with the other channel. In this study we chose to calculate the mean value of the Mander's coefficient for the Munc18-1 channel over mGluR4 channel and, thus, to evaluate the proportion of Munc18-1 signal which colocalizes with the mGluR4 signal ($r = x \pm$ S.E.) (18).

Affinity Chromatography with Munc18-1—Recombinant Munc18-1 (3 mg of protein at 1 mg/ml in a buffer containing 100 mM $NaHCO_3$, 500 mM NaCl) was covalently bound to 2.5 ml of CNBr-activated Sepharose 4B resin (GE Healthcare) according to manufacturer's instructions. Proteins were extracted from cerebellum of 30–37-day-old male Sprague-Dawley rats with buffer containing 1% Triton X-100, 50 mM NaCl, 25 mM HEPES-NaOH, pH 8.0, 0.5 mM EDTA, 0.25 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 0.01 mg/ml E-64, 0.05 mg/ml antipaïn, 1 mM Na_3VO_4, and anti-phosphatases mixture (Sigma) with a ratio of 0.1 ml/10 mg of tissue. The homogenate was centrifuged at 14,000 × *g* for 20 min at 4 °C. The supernatant (5 mg of proteins) was incubated in a batch with the resin for 1 h at 23 °C. Resin was washed with 30 ml of 1% Triton X-100, 50 mM NaCl, 25 mM HEPES-NaOH, pH 8.0, 0.5 mM EDTA buffer. Elution was performed with 18 ml of buffer containing 1% Triton X 100, 500 mM NaCl, 25 mM HEPES, 0.5 mM EDTA, pH 8.0. Fractions (3 ml) were collected and analyzed on 10% SDS-PAGE followed by silver nitrate staining. In fractions of interest, the concentration of Triton X-100 was reduced to 0.02% using Bio-Beads® SM (Bio-Rad). These fractions were pooled and concentrated with Centricon devices (UFC201024PL, Millipore), loaded on 10% SDS-PAGE gel, and analyzed by Western blot, as described thereafter. A control was performed using the resin alone (without Munc18-1) treated in the same conditions.

Affinity Chromatography with Peptides Corresponding to mGluR4 Cytoplasmic Parts—For affinity chromatography experiments, five peptides of the intracellular part of mGluR4 were synthesized (GeneCust, Luxembourg): the full-length C-terminal peptide AI-59 (AKRKRSLKAVVTAATMSNKFTQKGNFRPNGEAKSELCENLETPALATKQTYVTYTNHAI), truncated cytoplasmic parts, namely AN-23 (AEQNVPKRKRSLKAVVTAATMSN), VN30 (VVTAATMSNKFTQKGNFRPNGEAKSELCEN), and GI-30 (GEAKSELCENLETPALATKQTYVTYTNHAI), and the second intracellular loop NQ-25 (NRIYRIFEQGKRSVSAPRFISPASQ). Peptides were solubilized in 200 mM $NaHCO_3$, 500 mM NaCl at a concentration of 4 mg/ml. For peptides containing cysteine residues, cysteines were reversibly blocked using 5,5′-dithiobis(nitrobenzoic acid) as mentioned in Goyer *et al.* (19) to avoid the formation of peptide dimer during binding process. A protocol of peptide binding (4 mg) on 2 ml of slurry *N*-hydroxy succinimide activated Sepharose 4 Fast Flow resin (GE Healthcare) was established according to the manufacturer's instructions.

Proteins were extracted from the cerebellums of 30–37-day-old male Sprague-Dawley rats with buffer containing 50 mM NaCl, 25 mM HEPES-NaOH, pH 8.0, 0.5 mM EDTA, 0.25 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 0.01 mg/ml E-64, 0.05 mg/ml antipaïn, 1 mM Na_3VO_4, and an anti-phosphatases mixture (Sigma) with a ratio of 100 μl/10 mg of tissue. The homogenate was centrifuged at 14,000 × *g* for 20

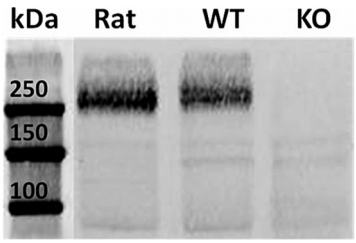

FIGURE 1. **Antibodies used for co-IP of mGluR4 specifically recognized the receptor.** 50 μg of cerebellar proteins from rat and mGluR4 knock-out (*KO*) or wild type (*WT*) mice were extracted and loaded on 10% SDS-PAGE, then probed with C-terminal anti-mGluR4 antibodies (Invitrogen).

min at 4 °C. The supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C, whereas the pellet was solubilized in the same buffer supplemented with 1% Triton X-100 and centrifuged again at 14,000 × *g* for 20 min at 4 °C. Both extracts were used for affinity chromatography experiments. Samples (3–3.5 mg of proteins at a concentration of 0.5 mg/ml) were loaded on the resin, and the column was washed with 10 ml of buffer. Elution was performed with 7 ml of buffer containing 25 mM HEPES-NaOH, pH 8.0, 500 mM NaCl, 0.5 mM EDTA. Eluates were concentrated with Centricon devices (UFC201024PL, Millipore) and loaded on 10% SDS-PAGE gel for Western blot. Controls were performed with the same resin without peptide.

Immunoblot Analysis—After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) or to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences) for 1 h at 100 V with buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS. The membrane was incubated for 1 h in Odyssey buffer (LICOR Biosciences, NE) and TBS (50 mM Tris, 150 mM NaCl, pH 7.5) at the ratio 1:1 as blocking buffer. Analysis of immunoblot was carried out by the Odyssey® Infrared Imaging system (LICOR). Secondary antibodies labeled with near-infrared dyes were used for protein detection; IRDye 800 has a λ_{max} of 780 nm and a $\lambda_{emission}$ of 815 nm, and IRDye 680 has a λ_{max} of 683 nm and a $\lambda_{emission}$ of 710 nm. Parameters such as dilution of the antibody, temperature, and incubation time were optimized for each antibody.

RESULTS

We verified the specificity of anti-mGluR4 antibodies (Invitrogen) used in co-immunoprecipitation (co-IP) by Western blot analysis. These experiments were performed on cerebellum extracts from rat, wild type mice, and mGluR4 knock-out mice in which the gene coding for the receptor had been deleted (5) (Fig. 1). A band at 250 kDa (likely corresponding to dimeric mGluR4) was observed both in rat and wild type mice extracts but not in those from mGluR4 KO mice. Similar results were obtained with several commercial anti-mGluR4 antibodies (data not shown). In co-IP experiments, an additional band at 100 kDa was observed, as shown in Fig. 2*a*, corresponding to the monomeric form of mGluR4. These results demonstrate that antibodies used for co-IP and Western blot are specific for mGluR4. It should be noted that when samples were heated before loading on SDS-PAGE, the receptor aggregated and remained in the stacking gel. For mGluR4 to enter the resolving gel and, as such, migrate properly, the samples should not be preheated.

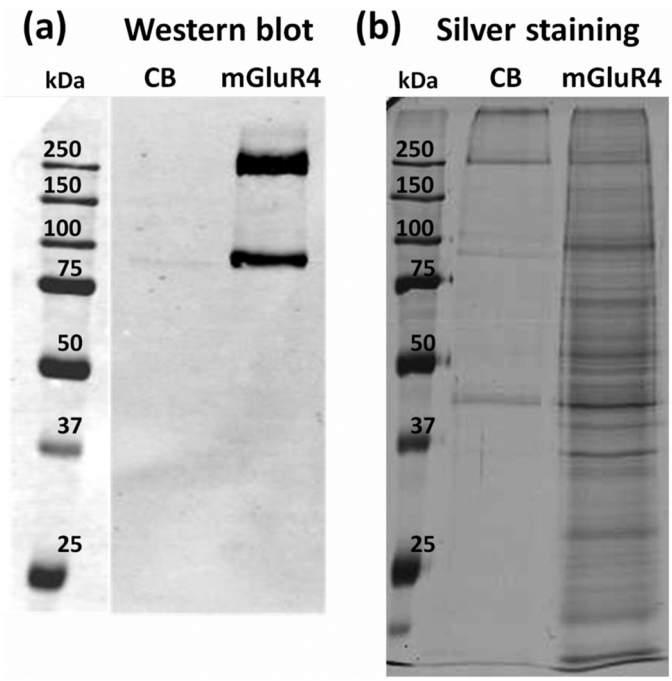

FIGURE 2. **Separation by SDS-PAGE of rat cerebellum proteins co-immunoprecipitated using anti-mGluR4 antibodies.** Immunoprecipitation with anti-mGluR4 or anti-CB antibodies was performed as described under "Experimental Procedures." After elution, co-immunoprecipitated proteins were separated on 10% SDS-PAGE and revealed by Western blot using anti-mGluR4 antibodies (Millipore) (*a*) and silver nitrate staining ($n = 3$) (*b*).

An assay was performed to determine whether concentration of glutamate, the endogenous agonist, was sufficient to activate mGluR4 in our experimental conditions. Glutamate was measured at a concentration of 1 mM (±0.25 mM, an average of three separate experiments) in the supernatant used for co-IP (data not shown). Under these conditions, mGluR4 should be activated and thus able to interact with its putative transduction pathway partners as the EC_{50} of glutamate for this receptor is 3–20 μM (20).

To define the optimal working buffer for co-IP experiments, two detergents, Triton X-100 (TX-100) and digitonin, were used at different concentrations, 0.5–1 and 1–2%, respectively, and at two different pHs (7.4 and 8.0). The relative amount of native mGluR4 extracted from rat cerebellum in each buffer condition was assessed by Western blot. Co-immunoprecipitation was then performed using anti-CB antibodies as a negative control as described in Farr *et al.* (16). The proteins that co-eluted either with anti-mGluR4 or anti-CB antibodies were separated on SDS-PAGE and stained with silver nitrate.

TX-100 at a concentration of 1% and at pH 8.0 provided the most efficient extraction of mGluR4 but failed to give a significant differential pattern (data not shown). On the other hand, a buffer containing 2% digitonin at pH 8.0 extracted a lower amount of receptor than 1% TX-100 but allowed successful immunoprecipitation of mGluR4 with its putative partners, indicating that this buffer is mild enough to maintain protein interactions (Fig. 2*a*). Indeed, numerous proteins were co-immunoprecipitated with anti-mGluR4 antibodies as opposed to the control antibodies (Fig. 2*b*). In addition, the efficiency of co-IP was assessed by Western blot, showing that all of the

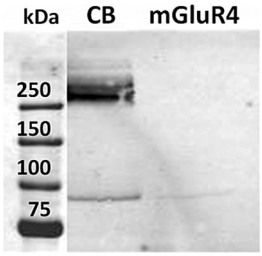

FIGURE 3. **Control of the mGluR4 co-IP efficiency**. Immunoprecipitation with anti-mGluR4 or anti-CB antibodies was performed as described under "Experimental Procedures." Proteins that were not co-immunoprecipitated and remained in the flow-through after co-IP were loaded on 10% SDS-PAGE and analyzed by Western blot (50 μg of proteins/lane) using anti-mGluR4 antibodies to validate the complete immunoprecipitation of the receptor.

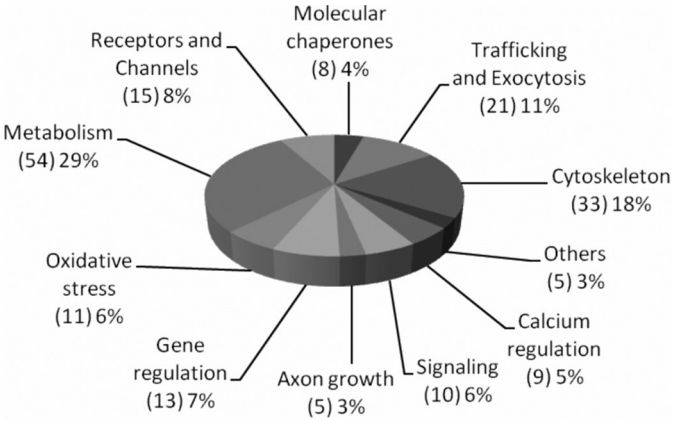

FIGURE 4. **Classification of the proteins identified in the three co-IP experiments using anti-mGluR4 antibodies.** Proteins were identified by LC-MS/MS and classified within 11 families. The number of proteins is indicated in *parentheses*.

mGluR4 receptor present in the supernatant had been immunoprecipitated with the anti-mGluR4 antibodies under these conditions (Fig. 3).

Three separate experiments were performed, and each resulted in similar differential patterns after silver nitrate staining. For protein identification, the whole SDS-PAGE lanes were cut into small bands, submitted to tryptic digestion, and analyzed independently by nanoLC-MS/MS. The eluates obtained with anti-mGluR4 and control antibodies were treated in the same way. Proteins identified in these control bands were considered as nonspecific interacting proteins and, therefore, removed from the list of mGluR4 partners. As expected, the two bands that gave a specific signal in Western blot experiments at 250 and 100 kDa were identified as mGluR4 by mass spectrometry. We identified a total of 184 proteins (mGluR4 and 183 potential partners) in the data pooled from these three experiments (supplemental Table 1). Fig. 4 shows the classification of these proteins according to their principal cellular functions such as proteins involved in metabolism (*54*), cytoskeleton (*33*), trafficking and exocytosis (*21*), receptors and channels (*15*, including mGluR4), gene regulation (*13*), oxidative stress (*11*), signaling (*10*), Ca^{2+} regulation (*9*), molecular chaperones (*8*), axon growth (*5*), and non-classified proteins (*5*). Specifically, our experimental approach identified several proteins that have been previously described as partners of mGluR4, such as β-spectrin, myosin, clathrin, microtubule-associated protein, and calcium-binding protein 1 (see supplemental Table 1). It should be noted that no protein of the post-synaptic element, such as abundant post-synaptic density proteins, was found in any of the three experiments, suggesting that co-IP was rather specific.

We then focused on proteins belonging to the membrane presynaptic vesicular trafficking proteins family because mGluR4 is localized at the presynaptic zone (21), close to the exocytosis machinery and is known to negatively regulate synaptic neurotransmission (8). These proteins are shown in Table 1. Among them, Munc18-1 had already been described as a potential mGluR4 partner in co-IP experiments with synaptosomal preparations and GST pulldown experiments (13). In addition, we identified presynaptic vesicle proteins such as synapsins I and II, synaptic vesicle glycoprotein 2A (SV2A), which could regulate the initial steps of exocytosis, target-SNAREs such as SNAP-25 and Syntaxin-1B, ATPase *N*-ethylmaleimide-sensitive factor, which dissociates SNARE complex and permits recycling of these proteins after membrane fusion, and α-synuclein, which is involved in exocytosis regulation (22, 23). Finally, proteins from the Ras-protein superfamily such as Rab, Rac, and Rho, which are involved in vesicular trafficking (24), were also found in our study. The presence of Munc18-1, SNAP-25, synapsins I and II, and syntaxin in the eluates of co-IP experiments was validated by Western blot analysis as shown in Fig. 5.

To confirm native interactions between presynaptic vesicular trafficking proteins and mGluR4, reverse co-IPs were performed on rat cerebellum extracts with antibodies directed against Munc18-1, Syntaxin, and SNAP-25. However, this receptor could not be detected in co-IP eluates. Although mGluR4 is relatively abundant in the cerebellum compared with other brain structures, it remains very weakly expressed in comparison with exocytosis and trafficking proteins. Therefore, the probability of detecting mGluR4 as a partner of Munc18-1, syntaxin, or SNAP-25 in reverse co-IPs was quite low.

To circumvent this limitation, we developed another reverse strategy to demonstrate that an exocytosis protein could target mGluR4. We performed affinity chromatography with recombinant Munc18-1. This protein has already been identified as a putative partner of mGluR4 but only when the C terminus of mGluR4 was the bait (13). Therefore, Munc18-1 was overexpressed in *Escherichia coli* as a polyhistidine-tagged protein, purified, and covalently bound on activated Sepharose matrix. For these experiments, rat cerebellar proteins were extracted with TX-100 to obtain the maximal amount of mGluR4. After loading the cerebellar extract onto the Munc18-1 column, retained proteins were eluted with a concentrated saline solution, and the presence of mGluR4 was investigated by Western blot analysis. A blank was performed under the same conditions using the resin alone. Native mGluR4 was specifically detected in the eluates from the Munc18-1 column, which confirms the ability of the native receptor to interact with this protein (Fig. 6). In addition, proteins such as syntaxin and SNAP-25, which have already been described as Munc18-1 partners (25–27), and synapsin I, were also present in the eluates. The experiment was performed twice and gave similar results. Together with

TABLE 1

mGluR4 partners involved in exocytosis and cellular trafficking

Shown is a list of trafficking and exocytosis proteins identified by mass spectrometry after co-IP. 9 of 21 identified proteins (in bold) are required for synaptic exocytosis (M_r, molecular weight; pI, isoelectric point). NSF, *N*-ethylmaleimide sensitive fusion protein.

Accession Number (UniProt KB)	Gene name	MOWSE score	Sequence coverage	Number of unique peptides	M_r	pI	Description	Number of experiments
			%					
P61765	**STXB1_RAT**	**329**	**9**	**5**	**67,569**	**6.3**	**Munc18 - 1**	**3**
Q9QUL6	**NSF_RAT**	**593**	**14**	**10**	**82,653**	**6.5**	**NSF**	**1**
Q6NYB7	RAB1A_RAT	99	9	2	22,977	6	Rab-1A	1
P63012	**RAB3A_RAT**	**138**	**11**	**2**	**24,970**	**4.9**	**Rab-3A**	**1**
B0BNK1	B0BNK1_RAT	219	17	3	23,639	8.6	Rab-5C	1
Q9WVB1	RAB6A_RAT	132	11	2	23,645	5.4	Rab-6A	1
Q5RKJ9	Q5RKJ9_RAT	212	17	3	22,755	8.6	Rab-10	2
P50398	GDIA_RAT	384	17	6	50,537	5	Rab GDI α	2
Q6RUV5	RAC1_RAT	98	9	2	21,826	7.5	p21-Rac1	1
Q5XI73	GDIR1_RAT	135	15	2	23,407	5.2	Rho GDI-1	1
P60881	**SNP25_RAT**	**141**	**14**	**2**	**23,315**	**4.7**	**SNAP-25**	**1**
P09951	**SYN1_RAT**	**241**	**8**	**4**	**73,988**	**9.8**	**Synapsin I**	**2**
Q63537	**SYN2_RAT**	**113**	**4**	**2**	**63,457**	**7.6**	**Synapsin II**	**1**
Q02563	**SV2A_RAT**	**128**	**3**	**2**	**82,661**	**5.5**	**SV2A**	**1**
P29101	**SYT2_RAT**	**147**	**4**	**2**	**47,210**	**8.2**	**Synaptotagmin-2**	**1**
P61265	**STX1B_RAT**	**144**	**10**	**2**	**33,245**	**5.3**	**Syntaxin-1B**	**2**
P37377	SYUA_RAT	176	18	2	14,515	4.8	α-Synuclein	1
Q63754	SYUB_RAT	207	18	3	14,504	4.5	β-Synuclein	1
Q05140	AP180_RAT	265	5	4	91,851	4.8	AP180	1
P11442	CLH_RAT	887	8	13	191,468	5.5	Clathrin heavy chain 1	3
P21575	DYN1_RAT	575	11	9	92,376	6.3	Dynamin 1	2

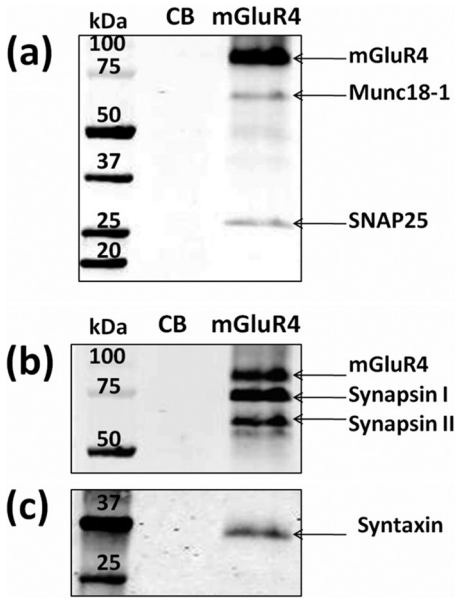

FIGURE 5. **Exocytosis proteins co-immunoprecipitated with mGluR4.** Proteins co-immunoprecipitating with anti-mGluR4 or anti-CB antibodies were revealed by Western blot with antibodies directed against Munc18-1 and SNAP-25 (*a*), synapsins I and II (*b*), and syntaxin (*c*) (the top of the blot membrane exhibiting the 250-kDa band has been cut due to the saturation of the signal).

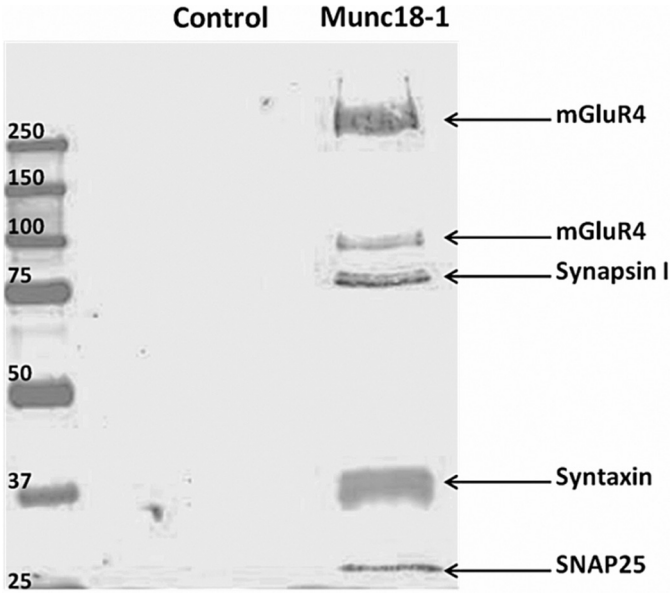

FIGURE 6. **Recombinant Munc18-1 interacted with native mGluR4 from rat cerebellum in an affinity chromatography experiment.** After affinity chromatography on recombinant Munc18-1 covalently grafted to Sepharose resin, proteins were eluted and analyzed by Western blot using antibodies directed against mGluR4, synapsin I, syntaxin, and SNAP-25. The blank (control) corresponds to the Sepharose matrix alone ($n = 2$).

co-IP results, these data suggest that Munc18-1, SNAP-25, synapsins, syntaxin, and mGluR4 form stable complexes.

To further characterize Munc18-1 and mGluR4 interaction, we investigated their co-localization in HEK293 cells. Munc18-1 expressed alone was highly concentrated in the cytoplasm (Fig. 7*a*), which is consistent with previous observations (28), whereas mGluR4 expressed alone was mainly observed at the cell membrane (Fig. 7*b*). Capping experiments with mGluR4 allowed us to specifically visualize membrane expression of the receptor (Fig. 7*c1*). We used the technique of co-capping to detect an association between Munc18-1 and mGluR4. We showed that among the population of Munc18-1 clusters (Fig. 7*c2*), a significant proportion colocalized with caps of mGluR4 (Fig. 7*c3*), which was supported by calculation of Mander's coefficient (r) ($r = 0.27 \pm 0.07$, $n = 10$, see "Experimental Procedures"). Thus, the localization of Munc18-1 toward mGluR4 caps within the plasma membrane was quite striking and strongly supported our proteomic and biochemical data concerning Munc18-1/mGluR4 interaction.

The interaction domain of mGluR4 with exocytosis proteins was further studied by affinity chromatography experiments. Peptides corresponding to the second intracellular loop and to the entire or partial C-terminal tail of the receptor were synthesized and covalently fixed on a Sepharose column (Table 2 and

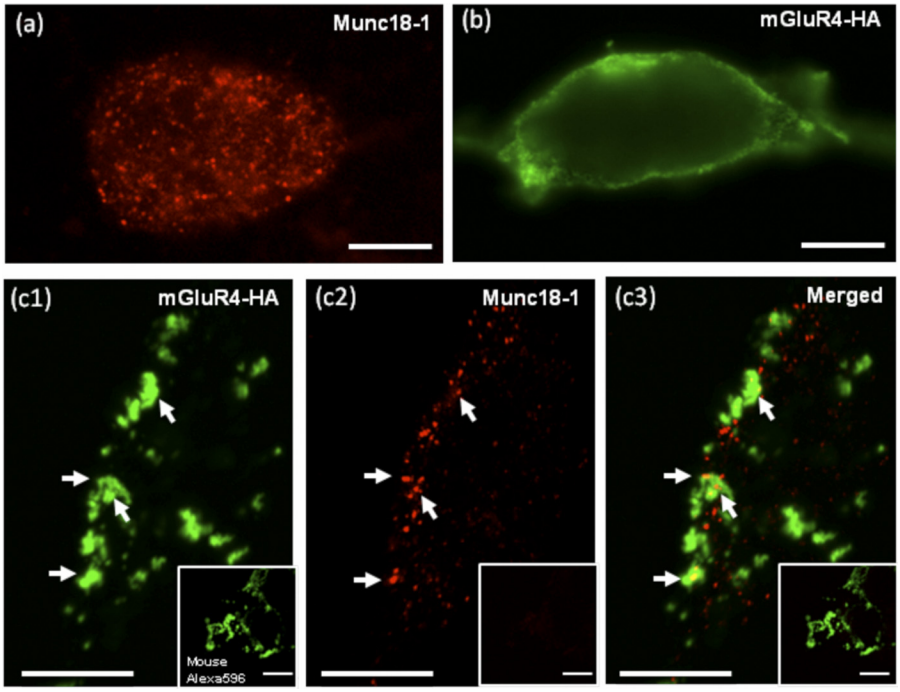

FIGURE 7. **mGluR4 and Munc18-1 colocalized in HEK 293 cells.** *a*, transient expression of Munc18-1 protein (mainly cytoplasmic) in HEK 293 cells labeled with mouse anti-Munc18-1 primary antibody and detected with Alexa488 goat anti-mouse secondary antibody (*red*) is shown. *b*, transient expression of HA-tagged mGluR4 (mainly within the plasma membrane) in HEK 293 cells labeled with chicken anti-HA primary antibody and detected with Alexa446 donkey anti-chicken secondary antibody (*green*) is shown. *c*, co-capping experiments of mGluR4 and Munc18-1 is shown. *c1*, mGluR4 capping was induced by cross-linking with HA antibody on the cell surface and detected with Alexa488-labeled secondary antibody (*green*). Alexa596 secondary antibody was used as a control to ensure no cross-reactivity with anti-HA antibody, used to detect HA-tagged mGluR4 (*c1*, *inset*). *c2*, Munc18-1 labeled with anti-Munc18-1 antibody and detected with Alexa546-labeled secondary antibodies (*red*) is shown. Note that no cross-reactivity was observed (*c2*, *inset*), although mGluR4 was well capped (*c1*, *inset*). *c3*, Munc18-1 was recruited to mGluR4 caps and thus partially co-localized with mGluR4 at the plasma membrane (*c1–3*, *white arrows*). Note that co-capping of mGluR4 and Munc18-1 was observed on 79% of mGluR4-expressing cells ($n = 210$). *Bar*, 10 μm.

TABLE 2

Characteristics of mGluR4 intracellular peptides used in affinity chromatography

AI-59 corresponds to the C-terminal tail of mGluR4, except that the first Pro was changed into Ala. AN-23, VN-30, and GI-30 correspond to overlaying fragments of the full length C-terminal tail of mGluR4 (from proximal part to distal part respectively). NQ-25 corresponds to the second intracellular loop.

Name of peptide	Sequence	M_r	Isoelectric point
AI-59	AKRKRSLKAVVTAATMSNKFTQKGNFRPNGEAKSELCENLETPALATKQTYVTYTNHAI	6555	9.92
AN-23	AEQNVPKRKRSLKAVVTAATMSN	2499	11.10
VN-30	VVTAATMSNKFTQKGNFRPNGEAKSELCEN	3272	8.15
GI-30	GEAKSELCENLETPALATKQTYVTYTNHAI	3296	4.91
NQ-25	NRIYRIFEQGKRSVSAPRFISPASQ	2908	11.55

Fig. 8). Cerebellum proteins extracted either with or without detergent (1% TX-100) were used for affinity chromatography using concentrated saline solution for elution. The presence of some exocytosis proteins of interest in the different eluates was verified by Western blot.

Affinity chromatography experiments performed with the entire C-terminal peptide, called AI-59, validated the interaction of Munc18-1, syntaxin, and synapsin I with mGluR4 (Fig. 9*a*). The same proteins were found to also interact with the second intracellular loop of the receptor called NQ-25 (Fig. 9*b*). By contrast, SNAP-25 was not detected using this approach. All the results from affinity chromatography experiments were analyzed by Western blot are summarized in Table 3. To obtain supplemental information on the interaction domains of mGluR4 with its binding partners, we used truncated fragments of the AI-59 peptide, namely AN-23, VN-30, and GI-30, from the N terminus to the C terminus, respectively. Munc18-1 was found to interact with the AN-23 peptide (Fig. 9*c*), whereas VN-30 and GI-30 peptides showed no interaction (Table 3). The positive signal with AN-23 was observed on proteins extracted both with and without detergent, although it was more intense with the use of detergent (1% TX-100). The experiments were performed at least twice and gave similar results. This affinity chromatography approach thus allowed us to validate the interaction of several exocytosis proteins with mGluR4 with potential binding sites on the second intracellular loop and/or on the C-terminal tail of the receptor.

DISCUSSION

A total of 183 proteins was found to interact with native mGluR4 in our co-IP experiments. Proteins were classified in different families according to their cellular function. Among them, metabolism and cytoskeleton proteins account for nearly 50% of the proteins identified. Due to their high abundance in the synapse, these protein groups are usually found on pre- or post-synaptic receptors in proteomic experiments. This is the

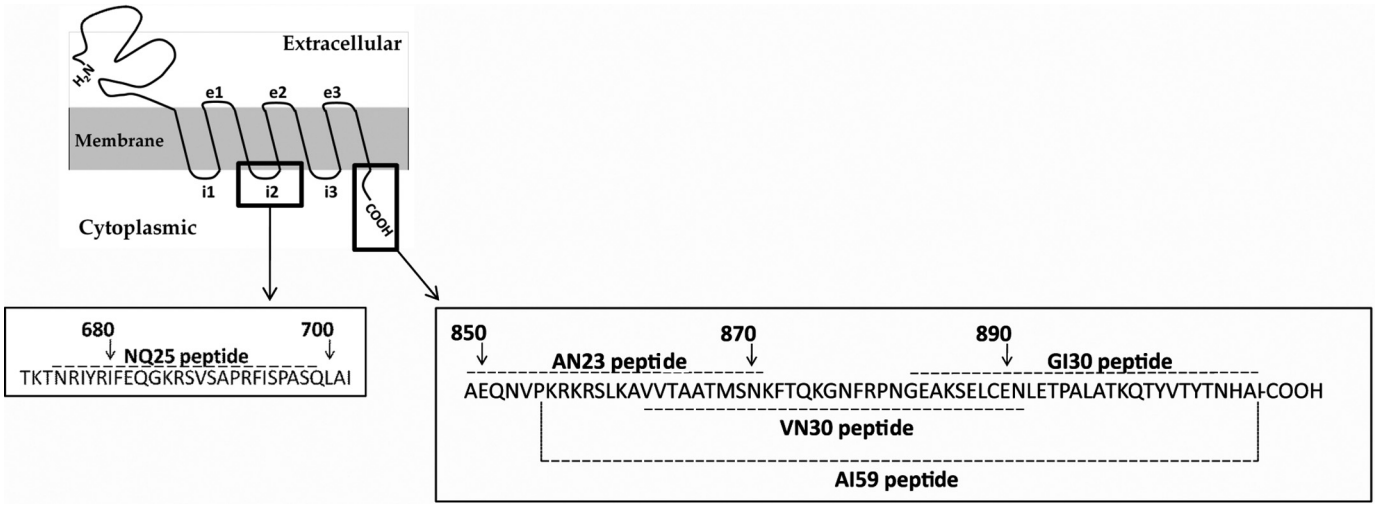

FIGURE 8. **Schematic structure of mGluR4 and representation of peptides used for affinity chromatography.** The receptor hmGluR4 is a G-protein-coupled receptor with a large N-terminal domain, seven transmembrane domains, and several cytoplasmic areas: three intracellular loops and a short C-terminal tail. The peptide AI-59 was modified by replacing the first Pro by an Ala.

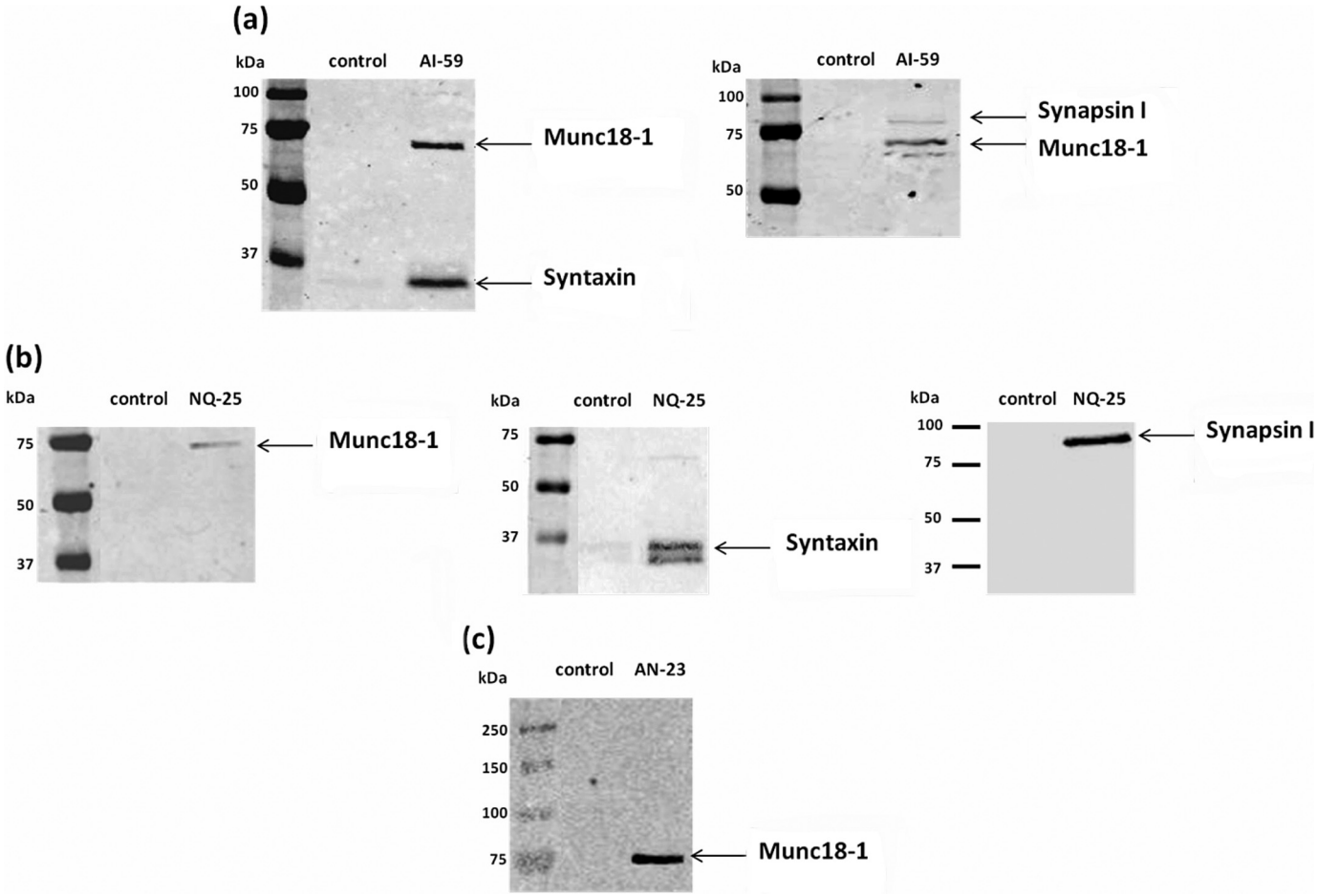

FIGURE 9. **Munc18-1, synapsin I, and syntaxin interacted with the cytoplasmic C-terminal tail and with the second intracellular loop of mGluR4.** A Western blot was performed after affinity chromatography with peptides AI-59 corresponding to the full-length cytoplasmic tail of mGluR4 (*a*), NQ-25 corresponding to the second intracellular loop of mGluR4 (*b*), and AN-23 corresponding to the proximal part of the C-terminal tail of mGluR4 ($n = 2$ for each peptide) (*c*).

case for example in studies targeting $Ca_v2.2$ or N-type calcium channels, which are involved in neurotransmitter release (29), or the GluR4 subunit of post-synaptic AMPA receptor (30). In all cases co-immunoprecipitated proteins are found specifically in the synaptic compartment containing the receptors. That was also the case in our study, as no post-synaptic protein was identified. Several partners already proposed in other studies (9, 12) have been found in our work, such as β-spectrin, myosin,

TABLE 3

Proteins interacting with cytoplasmic domains of mGluR4 in peptide affinity chromatography experiments, revealed by western blot analysis

+, Antibodies recognized the protein; −, antibodies were tested but the protein was not found.

Name of the protein	AI-59 (full-length)	AN-23	VN-30	GI-30	NQ-25 (second intracellular loop)
Munc18-1	+	+	−	−	+
SNAP-25	−	−	−	−	−
Synapsin I	+	−	−	−	+
Syntaxin	+	−	−	−	+

clathrin, adaptor protein complex, calcium-binding protein 1 (see supplemental Table 1), and should be further studied. In contrast, some other putative interacting proteins (syntenin, glutamate receptor interacting protein) highlighted in heterologous systems (11) were not identified in our study. Although protein interacting with c-kinase 1 (PICK1) was found to interact with mGluR4 and mGluR7 in heterologous systems (31), we did not identified this protein as a native mGluR4 partner. In cultured cerebellar granule cells, the PICK1/mGluR7 interaction leads to protein kinase C (PKC) activation (32), indicating a functional role of PICK1 in cellular mechanisms of mGluR7. This activation induces inhibition of presynaptic P/Q-type voltage-gated calcium channels, leading to reduction of glutamatergic synaptic transmission (32). Recently, it appears that this inhibition would involve the phosphorylation of SNAP-25 on Ser-187 by PKC (33, 34). These results show the tight link between mGluR7 and scaffold/exocytosis proteins in the control of neurotransmission regulation via voltage-gated calcium channels. Although we did not identify these channels as interacting proteins of native mGluR4 in our study, we cannot exclude that the negative modulation of neurotransmission induced by mGluR4 activation could involve transient inhibition of presynaptic voltage-gated calcium channels. Indeed, the nature of the intermediate molecular players involved in the signaling cascades responsible for the transient inhibition of these channels remains an open question. Otherwise, we did not find any Homer protein in mGluR4 receptosome, which can be explained by their specific interactions with group I mGluRs. Due to the crucial roles of Homer proteins in membrane expression of mGluR5 (35) and in the functional coupling efficiency of group I receptors (16, 36), further studies should be performed to determine whether similar proteins could play such a role for mGluR4.

In this study we focused on exocytosis/trafficking proteins (21 proteins identified here) because of their proximity to mGluR4. Among trafficking proteins, 9 of the 21 identified proteins belong to exocytosis family. Proteins involved in the regulation of glutamate release were identified, such as synaptotagmin-2, which is a Ca^{2+} sensor involved in neurotransmitter release at central nervous system synapses (37). Recently, synaptotagmins were found to interact with SV2A (also identified in our experiments), and SV2A proteins would regulate their expression and internalization (38). Synapsins I and II, which may play a role in the stabilization of synaptic vesicles to maintain a pool of neurotransmitters available (39), were also identified in our experiments. Therefore, mGluR4 could be involved in the mobilization of synaptic vesicles through direct or indirect interactions with synaptotagmin-2, synapsins I and II, and SV2A.

Moreover, our proteomic approach revealed an interaction between mGluR4 and several native proteins of the SNARE complex. Indeed, syntaxin-1 and SNAP-25 (target SNAREs), which were identified in this study, associate with synaptobrevin-2 (VAMP-2, a vesicle SNARE) to create a ternary trans-SNARE complex. This complex is involved in the physical linkage of synaptic vesicles on the plasma membrane necessary for neurotransmitter release. We also identified α-synuclein, which is thought to facilitate the assembly of the trans-SNARE complex (23). Thus, mGluR4 may be able to regulate the SNARE complex assembly via its interactions, direct or indirect, with syntaxin, SNAP-25, and α-synuclein.

To gain insight into the interaction domains between mGluR4 and these exocytosis proteins, affinity chromatography experiments were performed on different peptides corresponding to the C-terminal tail (full-length or truncated in three fragments) and the second intracellular loop of mGluR4. Indeed, we confirmed the interaction of mGluR4 with Munc18-1, synapsin I, and syntaxin, both on the C-terminal peptide and on the second intracellular loop. Each of these proteins may bind to mGluR4 either directly via one or several binding sites or indirectly. Moreover, the AN-23 peptide, which is in close proximity to the membrane, was also able to retain Munc18-1. This result is consistent with the work of Nakanishi and co-workers (13) that demonstrated a direct interaction between Munc18-1 and mGluR4 via the membrane proximal region of C-terminal receptor using a GST pulldown approach. It is interesting to note that AN-23 is predicted to exhibit an α-helix secondary structure, whereas VN-30 and GI-30 peptides are less structured. This feature probably favors the implication of AN-23 sequence in native interactions. Finally, our affinity chromatography experiments highlighted the role of the second intracellular loop as an additional interaction domain of Munc18-1 with mGluR4. Notably, SNAP-25 was not found with this approach, although this protein belongs to the SNARE complex. Whatever, these results confirm that mGluR4 clearly interacts with a pool of proteins involved in exocytosis.

In particular, Munc18-1 was identified as a partner of native mGluR4 in all three co-IP experiments. In addition, recombinant Munc18-1 covalently grafted on Sepharose matrix was able to retain native mGluR4, which consolidated the co-IP results and suggests a direct physical interaction between Munc18-1 and mGluR4, as already reported by Nakajima *et al.* (13), for recombinant Munc18-1 and C-terminal mGluR4 constructs. Moreover, we demonstrated that these proteins colocalized at the plasma membrane in HEK293 cells. Studies on SNAREs complexes reconstituted in liposomes have defined a facilitator effect of Munc18-1 on synaptic fusion (40). *In vitro* experiments have shown that Munc18-1 interacts with the syn-

taxin-1 C-terminal part (41) but also with the SNARE complex itself (40, 42). Recently Munc18-1 has been shown to interact with mGluR7 and mGluR4 and play a role in short term plasticity (13). In our affinity chromatography experiments, Munc18-1 was found to interact with two different regions of mGluR4; that is, the proximal C-terminal and the second intracellular loop. Based on these results, it would be informative to further study the mechanisms of interaction between Munc18-1 and mGluR4 in the negative regulation of neurotransmitter release.

Our results suggest that mGluR4 could interact directly with exocytosis proteins with the purpose of limiting neurotransmitter release by sequestering one or more of these proteins. Whether these interactions correspond to direct physical interactions should be further investigated to better understand the molecular mechanisms involved in this process. Taken together our results suggest that modulation of synaptic transmission by group III mGluRs may be at least partly independent of the classical G-protein transduction pathway.

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