Differential expression of two novel Munc13 proteins in rat brain

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Munc13-1, a mammalian homologue of *Caenorhabditis elegans* unc-13p, is a presynaptic phorbol ester receptor that enhances neurotransmitter release. In the present study we analysed the regional, cellular and subcellular expression patterns in rat of two novel Munc13 proteins, Munc13-2 and Munc13-3. We demonstrate by hybridization *in situ* that Munc13-1 mRNA is expressed throughout the brain, whereas Munc13-2 mRNA is preferentially present in rostral brain regions, and Munc13-3 mRNA in caudal areas. In an analysis of subcellular brain fractions with isoform-specific antibodies, we show that the novel Munc13 proteins are enriched in synapses. Immunocytochemical examination of rat cerebellar sections indicates that Munc13-3, like Munc13-1, is concentrated in presynaptic terminals. Our results characterize Munc13 proteins as a family of neuron-specific, synaptic molecules that bind to syntaxin, an essential mediator of neurotransmitter release. Munc13-2 and Munc13-3 are expressed in a complementary fashion and might act in concert with Munc13-1 to modulate neurotransmitter release.

Key words: exocytosis, phorbol ester, presynaptic, transmitter release.

INTRODUCTION

The release of transmitter from neurons is mediated by exocytosis. Synaptic vesicles, the key organelles in this process, undergo a complex cycle of fission and fusion events that regulate the release process at the synapse. Vesicles are generated by budding from early endosomes and loaded with neurotransmitter. After a translocation process, they dock at the active zone and mature to a fusion competent state. In response to a rise in the intracellular Ca^{2+} concentration, fusion-competent vesicles release their content through exocytosis. Vesicular protein and membrane components are then retrieved by clathrin-mediated endocytosis and recycled directly or via early endosomes for a new round of release [1–6].

Munc13-1, a mammalian homologue of the *Caenorhabditis elegans unc-13* gene product [7], participates in the regulation of the synaptic vesicle cycle by second messengers [8]. It interacts with the N-terminus of syntaxin, an essential component of the synaptic release machinery involved in vesicle maturation and fusion [9], and with DOC2, a putative regulator of transmitter secretion [10]. Munc13-1 is a receptor for diacylglycerol and phorbol ester that translocates to the plasma membrane in response to ligand binding [8]. It is localized to presynaptic transmitter release sites, where it is thought to function in synaptic vesicle recruitment, docking or priming [8]. Munc13-1 is a functional presynaptic target of the diacylglycerol second-messenger pathway that acts in parallel with protein kinase C. When overexpressed presynaptically in *Xenopus* neuromuscular junctions, it serves as a diacylglycerol-dependent and phorbol ester-dependent enhancer of neurotransmitter release [8].

In addition to Munc13-1, two other Munc13 mRNA species are expressed in the rat central nervous system, but the respective native proteins, Munc13-2 and Munc13-3, have not been identified. Like Munc13-1, recombinant full-length Munc13-2 and Munc13-3 are receptors for phorbol ester and diacylglycerol that associate with membrane bilayers in response to ligand binding [8]. However, the roles of Munc13-2 and Munc13-3 in brain function and their relationship to the Munc13-1 isoform are unknown.

In the present study we cloned the full-length cDNA of Munc13-3 and performed a systematic comparison of rat Munc13 isoforms on the mRNA and protein levels. We demonstrate by hybridization *in situ* that the three Munc13 mRNA species are expressed in a neuron-specific manner and exhibit regional expression patterns in the brain that are partly overlapping and in part complementary to each other. Using specific antibodies we show that the respective proteins are concentrated in synapses, with Munc13-3, like Munc13-1, being localized to presynaptic terminals. Our results suggest that, depending on the synapse, Munc13-2 or Munc13-3 is co-localized with Munc13-1. The novel Munc13 isoforms might act in concert with Munc13-1 to modulate the release of neurotransmitter.

MATERIALS AND METHODS

Cloning of Munc13-3 and sequence analyses

A rat brain cDNA library in λZAPII (Stratagene, Heidelberg, Germany) was screened at high stringency with a probe derived from a partial Munc13-3 cDNA clone [7]. From 2.5×10^6 plaques, 16 positive clones were isolated. Rescued plasmids were sequenced by using the dideoxy chain-termination method with dye terminators on an Applied Biosystems 373 DNA sequencer (Applied Biosystems, Weiterstadt, Germany). Of the 16 isolated clones, 13 encoded parts of Munc13-3, and 3 represented artifacts. Overlapping clones containing the full-length coding region of Munc13-3 were isolated by rescreening the λZAPII library with uniformly labelled probes originating from the extreme 5′ and 3′ sequences isolated in the initial screen. A mammalian expression vector encoding full-length Munc13-3 was constructed from multiple overlapping cDNA clones in pcDNA3 (pcDNA-Munc13-3; Invitrogen, San Diego, CA, U.S.A.). Mammalian expression vectors for Munc13-1 (pcDNA-Munc13-1) and

Abbreviations used: GST, glutathione S-transferase; NMDA, *N*-methyl-D-aspartate; SNAP25, synaptosome-associated protein of 25 kDa.

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The Munc13-3 nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U24072.

Munc13-2 (pCMV-Munc13-2) have been published previously [7]. A human embryonic kidney cell line (HEK293) was transfected with these vectors by using published procedures [8].

Hybridization *in situ*

Male Wistar rats (8 weeks old) were anaesthetized and decapitated. The brains were removed and quickly frozen on solid CO_2. Sections were cut at 12 μm on a cryostat, thaw-mounted on silane-coated slides, fixed with 4 % (w/v) paraformaldehyde in PBS, dehydrated, and stored under ethanol until hybridization. Sections were hybridized for 15–20 h in hybridization buffer [50 % deionized formamide/10 % (v/v) dextran sulphate/0.3 M NaCl/30 mM Tris/HCl (pH 8)/4 mM EDTA/1 × Denhardt's/0.4 mg/ml polyadenylic acid/0.5 mg/ml denatured salmon sperm DNA] containing the radiolabelled probe at 0.01–0.02 ng/μl with specific radioactivities of $(4–8) \times 10^8$ d.p.m./μg. The oligonucleotide probes were generated with terminal transferase by using [^{35}S]dATPαS (NEN, Köln, Germany). Anti-sense oligonucleotides representing the following sequences were chosen as probes: bp 1480–1524 and 1927–1971 of Munc13-1 (GenBank accession no. U24070); bp 230–274 and 754–798 of Munc13-2 (GenBank accession no. U24071); bp 1535–1579 and 2365–2409 of Munc13-3 (GenBank accession no. U24072). Optimal oligonucleotide sequences (45-mers, 50–60 % GC, low hairpin and dimer formation probability) were selected by using Oligo 5.0 (NBI, Plymouth, MN, U.S.A.). In control experiments, hybridizations were performed with a 1000-fold excess of the respective unlabelled oligonucleotide. After hybridization, sections were washed twice with 1 × SSC/0.1 % (v/v) 2-mercaptoethanol at room temperature for 20 min, once with 1 × SSC/0.1 % (v/v) 2-mercaptoethanol at 57 °C for 45 min, once with 1 × SSC/0.1 % (v/v) 2-mercaptoethanol at room temperature for 5 min, and once with 0.1 × SSC/0.1 % (v/v) 2-mercaptoethanol at room temperature for 2 min. Sections were then dehydrated, air-dried and exposed to Kodak BioMax film (Eastman Kodak, New Haven, CT, U.S.A.) for 2 weeks at room temperature. Slides were then dipped in photographic emulsion (Kodak NTB3; Eastman Kodak), incubated for 6–9 weeks and developed in Kodak D-19 developer (Eastman Kodak) for 3.5 min. For analysis with bright-field optics and to confirm cytoarchitecture, sections were counterstained with Cresyl Violet.

Generation of antibodies against Munc13-1, Munc13-2 and Munc13-3

Polyclonal antibodies directed against Munc13-2 (AI 1) and Munc13-3 (AI 2) were generated with glutathione S-transferase (GST) fusion proteins as antigens. Recombinant GST–Munc13-2 and GST–Munc13-3 fusion proteins, representing parts of the unique N-termini of the proteins, were generated by using the following expression plasmids in the pGEX-KG vector [11]: pGEX-Munc13-2-211/212 (residues 1–305); pGEX-Munc13-3-113/114 (residues 291–568). The antisera were affinity-purified on fusion proteins coupled to CNBr-activated Sepharose (Pharmacia, Freiburg, Germany) [12]. A monoclonal antibody against Munc13-1 was generated in collaboration with BioGenes (Berlin, Germany) by using a fusion protein encoding GST in frame with residues 1399–1736 of Munc13-1 (pGEX-Munc13-1F) [8,9].

Immunocytochemistry

Adult mice were anaesthetized with ether and decapitated. Brains were immediately dissected and quickly frozen on solid CO_2. Sections 8 μm thick were cut on a cryostat for light-microscopic immunocytochemistry. Munc13-3 immunoreactivity was detected in unfixed material by the method of Rickmann and Wolff [13], with the affinity-purified antibodies to Munc13-3 (AI 2) at 0.5–1 μg/ml. Control experiments were performed with Munc13-3 knock-out mice (details available from the authors on request).

Yeast two-hybrid constructs

Yeast two-hybrid constructs of Munc13-1 (residues 1181–1736) [9], Munc13-2 (residues 1463–1985) and Munc13-3 (residues 1702–2204) were constructed in pLexN by using PCR. These constructs contain a highly homologous region that had previously been shown in Munc13-1 to interact with syntaxins 1B and 2 [9]. The resulting vectors express LexA fusion proteins with the respective Munc13 C-termini. Prey constructs for syntaxin 1B (residues 1–79) and syntaxin 2 (residues 53–227) in pVP16-3 (expressing VP16 fusion proteins) had previously been identified in yeast two-hybrid screens with a Munc13-1 C-terminal bait construct [9]. Prey expression vectors encoding full-length synaptosome-associated protein of 25 kDa (SNAP25) or synaptobrevin 2 (residues 1–96) in frame with VP16 have been published previously [9].

Miscellaneous

All chemicals were of the highest available purity and were purchased from standard sources. The following antibodies were used: monoclonal antibodies to syntaxin 1A/B [14], SNAP25 (Cl 71.2) [15], synaptobrevin 2 (Cl 69.1) [16], synaptotagmin 1 (Cl 41.1) [17], *N*-methyl-D-aspartate (NMDA) R1 (Cl 54.1) [18], synaptophysin (Cl 7.2) [19], α-tubulin (ICN, Eschwege, Germany), polyclonal antibodies against Munc13-1 [7,8], msec7 [20] and syntaxin 2 (I. Augustin and N. Brose, unpublished work) (gift from T. C. Südhof). SDS/PAGE and immunoblotting were performed with standard procedures [21,22]. Immunoprecipitations [18], subcellular fractionation of rat cortex [23], Ficoll gradient purification of synaptosomes [24] and purification of synaptic vesicles by controlled-pore glass bead chromatography [25] and immunobead precipitation [26] were performed in accordance with published procedures. β-Galactosidase assays in the yeast two-hybrid system were performed by the method of Rose et al. [27] on extracts from yeast strain L40 co-transformed with the indicated bait and prey constructs. Assays were performed at least twice (in triplicate) with essentially the same results.

RESULTS

Primary structure of Munc13-3

Multiple Munc13-3 cDNA clones were isolated in two screens of a rat brain cDNA library with a cDNA fragment of Munc13-3 originating from a previously isolated clone [7]. The full-length amino acid sequence of Munc13-3 was derived from multiple overlapping cDNA clones.

The primary structure of Munc13-3 consists of 2204 amino acid residues with a calculated molecular mass of 249 kDa. The domain structure of the protein is similar to that of other unc-13p-like proteins (Figure 1). The N-terminal 1085 residues share no homology with other known proteins, with the exception of a stretch of 54 residues with similarity to part of the Munc13-2 N-terminus (Figure 1). They are followed by a C_1 domain that is similar to the phorbol ester-binding regulatory domain of protein kinase C [8], and a C_2 domain that is also present in a large number of other proteins and whose function might range from Ca^{2+}-binding to mediating protein–protein interactions [7]. The

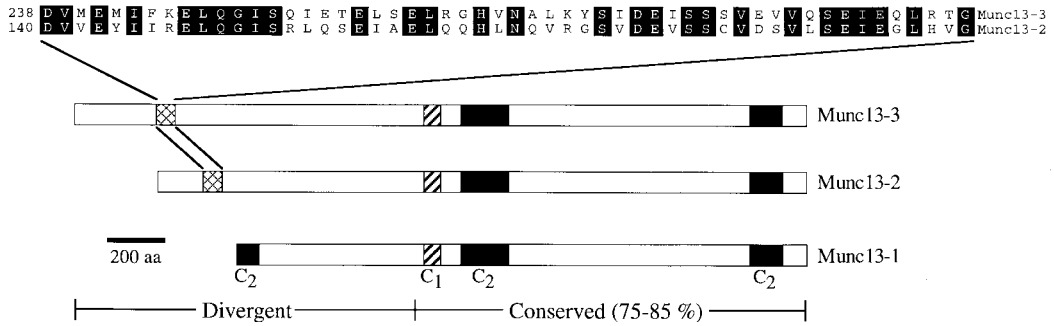

Figure 1 Structure of Munc13 isoforms

Munc13 isoforms are very large proteins (Munc13-1, 1735 residues, 196 kDa; Munc13-2, 1985 residues, 222 kDa; Munc13-3, 2205 residues, 249 kDa) containing C_1 and C_2 domains (hatched and black boxes respectively). Conserved and divergent sequences are indicated. The position of a short stretch of similar sequence in the otherwise unrelated N-termini of Munc13-2 and Munc13-3 is indicated by a cross-hatched box, and an alignment of the homologous sequences is shown at the top. The GenBank accession numbers are U24070, U24071 and U24072 for Munc13-1, Munc13-2 and Munc13-3 respectively. Abbreviation: aa, amino acid residues.

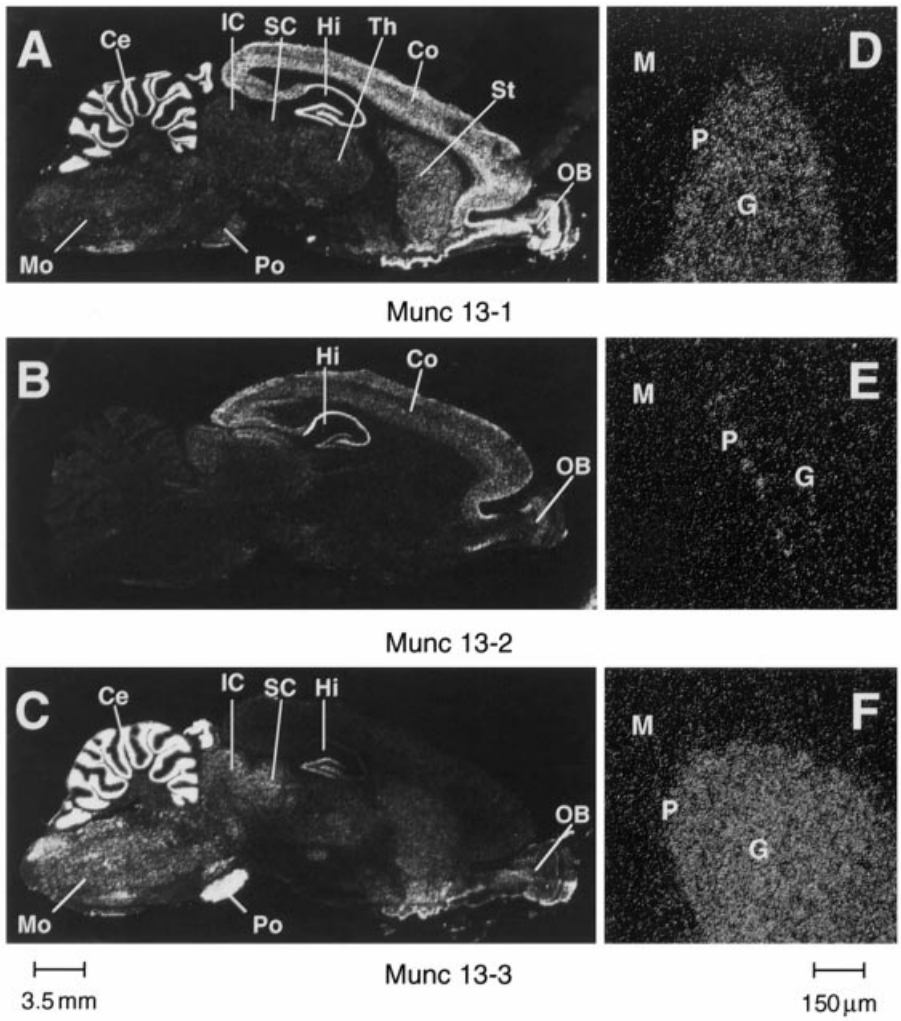

Figure 2 Expression of Munc13 mRNA species in rat brain

(**A**–**C**) Negative X-ray film images showing the distribution of Munc13 mRNA species in adult rat brain [Munc13-1 (**A**), Munc13-2 (**B**) and Munc13-3 (**C**)]. Negative controls with excess unlabelled oligonucleotides were devoid of signal (results not shown). Note the complementary expression patterns of Munc13-2 (rostral brain regions; CA regions of hippocampus) compared with Munc13-3 (caudal brain regions; dentate gyrus). Abbreviations: Ce, cerebellum; Co, cerebral cortex; Hi, hippocampus; IC, inferior colliculus; Mo, medulla oblongata; OB, olfactory bulb; Po, pons; SC, superior colliculus; St, striatum; Th, thalamus. Scale bar 3.5 mm. (**D**–**F**) Dark-field images of emulsion-dipped sections from rat cerebellum showing the differential distribution of Munc13 mRNA species in cerebellar neurons [Munc13-1 (**D**), Munc13-2 (**E**) and Munc13-3 (**F**)]. Munc13-1 and Munc13-3 are expressed in cerebellar granule cells as well as in Purkinje cells, whereas Munc13-2 is expressed only in Purkinje cells. Abbreviations: M, molecular layer; P, Purkinje cell layer; G, granule cell layer.

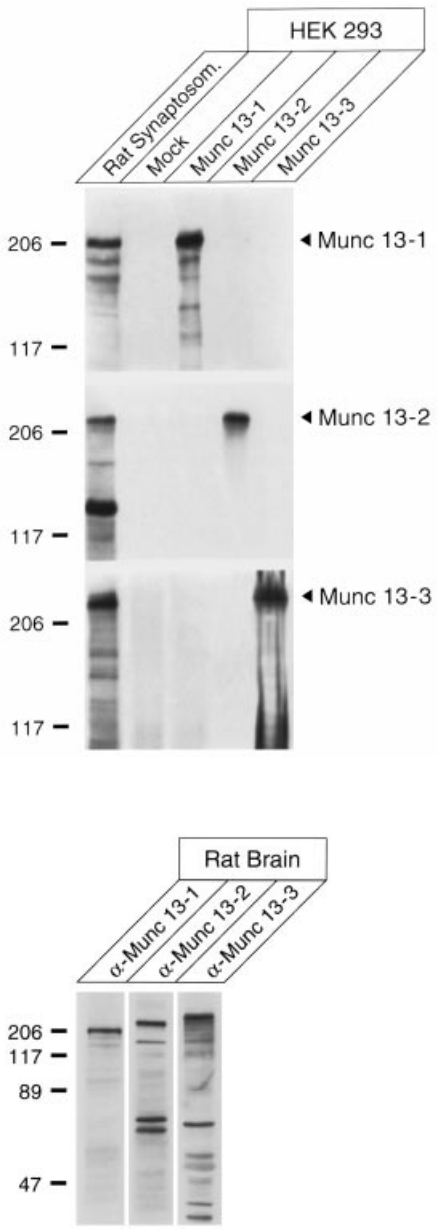

Figure 3 Characterization of antibodies against Munc13 isoforms

Upper panel: HEK293 cells transfected with the indicated full-length Munc13 expression constructs, mock-transfected HEK293 cells and rat brain synaptosomes [20 μg per lane; 7.5% (w/v) gel that was left running until the 117 kDa molecular mass marker reached the bottom of the gel] were analysed by being immunoblotted with monoclonal antibody 3H5 against Munc13-1 and affinity-purified antisera against Munc13-2 and Munc13-3 (AI 1 and AI 2). Note that the three antibodies are specific for the respective isoform they were raised against. The three Munc13 isoforms exhibit clearly distinct molecular masses (Munc13-1, 200 kDa; Munc13-2, 220 kDa; Munc13-3, 240 kDa). Full-length Munc13 isoforms are indicated by arrowheads. Lower panel: total rat brain homogenates [20 μg of protein per lane, 7.5% (w/v) gel] were analysed by being immunoblotted with monoclonal antibody 3H5 against Munc13-1 (α-Munc13-1) and affinity-purified antisera against Munc13-2 (α-Munc13-2) and Munc13-3 (α-Munc13-3) (AI 1 and AI 2). In addition to the respective full-length Munc13 isoforms, antibodies against Munc13-2 and Munc13-3 recognize unidentified protein bands at lower molecular masses (less than 89 kDa). In both panels the molecular masses of protein standards are indicated (in kDa) at the left.

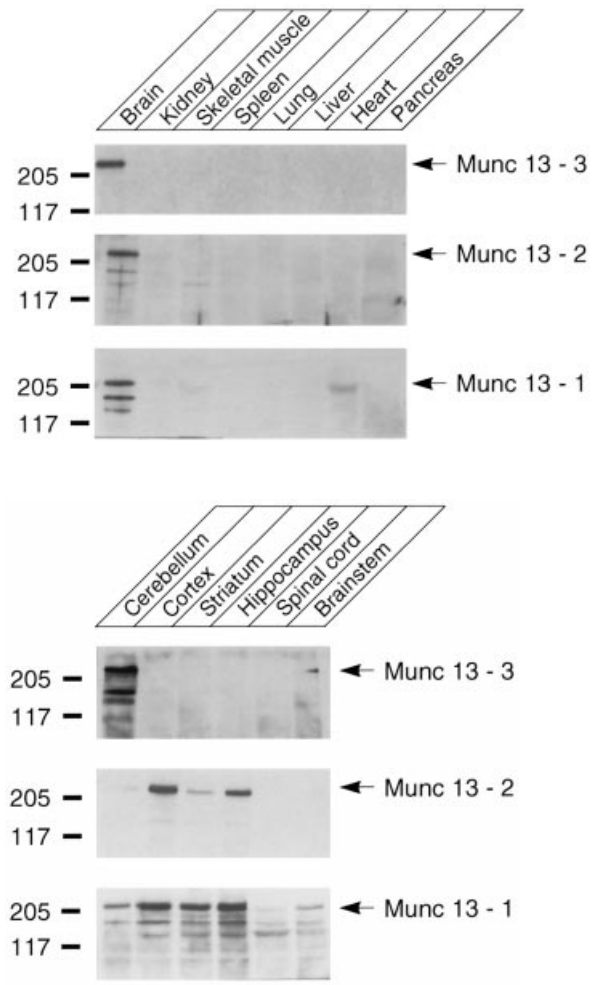

Figure 4 Tissue distribution of Munc13 proteins

Homogenates from the indicated tissues (upper panel) or brain regions (lower panel) (20 μg per lane) were analysed by being immunoblotted with isoform-specific antibodies. Lower bands in brain (upper panel) and brain regions (lower panel) were observed occasionally with anti-Munc13 antibodies and are assumed to represent proteolytic breakdown products as the same bands also appear in HEK293 cells overexpressing the respective isoforms but not in control cells (see Figure 3). Cross-reacting bands at 200 kDa in heart and skeletal muscle are caused by myosin. In both panels the molecular masses of protein standards are indicated (in kDa) at the left.

tandem C_1/C_2 domains are N-terminal to a stretch of 704 residues that is unique to, and highly conserved in, unc-13p-like molecules and that ends with a distantly related C_2 domain (Figure 1). Interestingly, the divergent N-termini of Munc13-2 and Munc13-3 are each encoded by a single large exon (not shown in Figure 1).

Localization of Munc13 mRNA species

On the basis of the novel Munc13-3 sequence and published sequences of Munc13-1 and Munc13-2 [7], oligonucleotides were designed for use in experiments with hybridization *in situ* on brain sections from adult rats. Two oligonucleotides for each of the three Munc13 isoforms were used; they exhibited essentially the same labelling patterns for the respective isoform. This labelling was abolished when an excess of unlabelled oligonucleotide was used in the hybridization mix (results not shown).

Detailed analyses of expression patterns were performed with one oligonucleotide for each Munc13 isoform (bp 1927–1971 for Munc13-1, bp 230–274 for Munc13-2, bp 1535–1579 for Munc13-

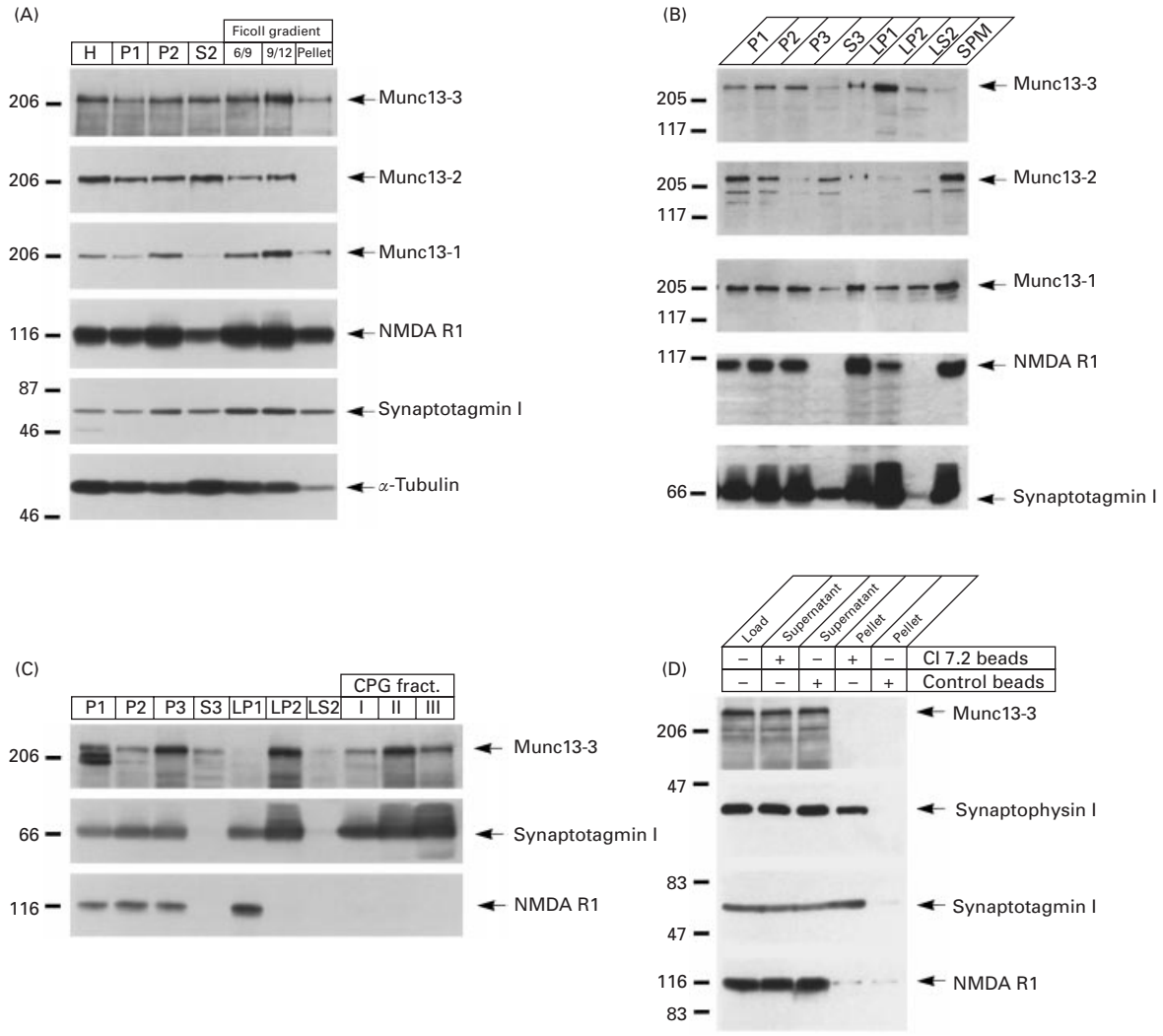

Figure 5 Subcellular localization of Munc13 proteins

(**A**) Ficoll gradient purification of synaptosomes from rat cerebral cortex. Homogenates (H) were separated into a nuclear fraction (P1), crude synaptosomes (P2) and a low-speed supernatant containing light membranes and cytosol (S2). High-purity synaptosomes (Ficoll gradient 9/12) were then isolated on a Ficoll step gradient and separated from a cruder synaptosomal fraction (Ficoll gradient 6/9) and a pellet enriched in mitochondria (Ficoll gradient pellet). Fractions (20 μg per lane) were analysed by being immunoblotted with antibodies specific for the indicated proteins. Note the co-enrichment of Munc13-1 and Munc13-3 with marker proteins (NMDA R1 and synaptotagmin 1) in the high-purity synaptosomal fraction. (**B**) Subfractionation of rat cerebral cortex by differential centrifugation. Fractions are designated as follows: P1, nuclear pellet; P2, crude synaptosomal pellet; P3, light membrane pellet; S3, cytosolic fraction; LP1, lysed synaptosomal membranes; LP2, crude synaptic vesicle fraction; LS2, cytosolic synaptosomal fraction; SPM, synaptic plasma membranes. Fractions (20 μg per lane) were analysed by being immunoblotted with antibodies specific for the indicated proteins. Note the co-enrichment of Munc13-1 and Munc13-2 with NMDA R1 in synaptic plasma membranes, and that of Munc13-3 with synaptotagmin 1 in the crude synaptic vesicle fraction (LP2). (**C**) Subfractionation of rat cerebral cortex by differential centrifugation and chromatography on controlled-pore glass beads. Rat cerebral cortex fractions were prepared as in (**B**) [see (**B**) for designation of fractions], and crude synaptic vesicles (LP2) were further purified by chromatography on controlled-pore glass beads. Three protein peaks were eluted from the column (CPG I, CPG II and CPG III), the last two of which (CPG II and CPG III) contained synaptic vesicles of very high purity. Fractions (20 μg per lane) were analysed by being immunoblotted with antibodies specific for the indicated proteins. Note the co-enrichment of Munc13-3 with the synaptic vesicle marker synaptotagmin 1. (**D**) Purification of synaptic vesicles by immunobead precipitation. Eupergit beads were coupled with a monoclonal antibody directed against synaptophysin (CI 7.2 beads) and used to immunoprecipitate synaptic vesicles from a precleared (centrifugation at 20 000 g_{max} for 60 min) rat cerebral cortex homogenate (load). Eupergit beads coupled with glycine were used as a control (control beads). The homogenate (load), supernatant and pellet of the immunoprecipitations were analysed by being immunoblotted with antibodies specific for the indicated proteins. Note that Munc13-3 did not co-purify with synaptotagmin or synaptophysin. In all panels the molecular masses of protein standards are indicated (in kDa) at the left.

3). Autoradiographs of hybridized rat brain sections revealed differential but overlapping expression patterns of Munc13 mRNA species. In each case, regions rich in glial cells (e.g. white matter of cerebral cortex and cerebellum) were unlabelled, indicating a neuron-specific expression of Munc13 isoforms (Figure 2). Munc13-1 mRNA is expressed throughout the brain, with highest levels in the olfactory bulb, striatum, cerebral cortex, hippocampus and cerebellum (Figure 2A). The expression of Munc13-2 was detected mainly in the olfactory bulb, cerebral cortex and hippocampus (Figure 2B). In contrast, Munc13-3 mRNA was found mainly in the cerebellum, brain stem and pons nuclei (Figure 2C). These results show that the three Munc13 isoforms are expressed differentially in rat brain. However, their expression overlaps such that at least two isoforms are expressed in any given region. A similar pattern of differential but overlapping expression of Munc13 isoforms was observed in the hippocampal formation. Here, strong labelling for Munc13-1 was observed in the CA regions and the dentate gyrus. Munc13-

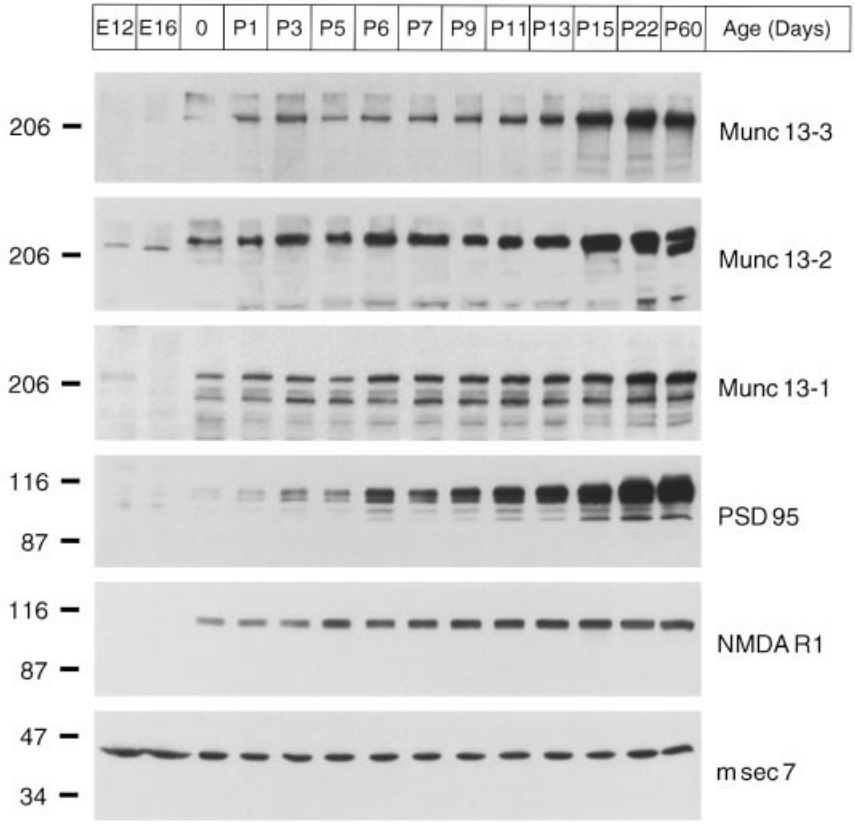

Figure 6 Expression of Munc13 proteins during development

Brains from rats of the indicated ages were homogenized and analysed (20 μg per lane) by being immunoblotted with antibodies specific for the indicated proteins. Abbreviations: E, embryonic day; P, postnatal day. Lower bands at 180 kDa (Munc13-1, all fractions) and at 200 kDa (Munc13-2, P60) were observed occasionally with anti-Munc13 antibodies and are assumed to represent proteolytic breakdown products because the same bands also appear in HEK293 cells overexpressing the respective isoforms but not in control cells (see Figure 3). The molecular masses of protein standards are indicated (in kDa) at the left.

2 mRNA was detected mainly in the CA regions, whereas Munc13-3 expression was largely restricted to the dentate gyrus (Figures 2A–2C).

To determine the cellular distribution of Munc13 mRNA species, we analysed emulsion-dipped sections. The cerebellum showed a differential distribution of Munc13 mRNA species at the cellular level. Within the cerebellum, Munc13-1 and Munc13-3 mRNA species were strongly expressed in both granule and Purkinje cells. In contrast, the low-level Munc13-2 labelling was restricted to Purkinje cells (Figures 2D–2F).

Characterization of Munc13-2 and Munc13-3 proteins

By using GST fusion proteins as antigens, we generated a monoclonal antibody directed against Munc13-1, and two polyclonal antisera directed against Munc13-2 (AI 1) and Munc13-3 (AI 2). The isoform specificities of these novel antibodies are shown in Figure 3. The three antibodies recognize respective antigens of clearly distinct molecular masses, corresponding to the predicted molecular mass based on primary structure. The monoclonal antibody 3H5 against Munc13-1 detected a major band of 200 kDa in rat brain and HEK293 cells expressing recombinant Munc13-1, whereas recombinant Munc13-2 or Munc13-3 was not detected. The affinity-purified polyclonal antibody AI 1 against Munc13-2 stained a major band of 220 kDa in rat brain and HEK293 cells expressing recombinant Munc13-2 but it did not detect recombinant Munc13-1 or Munc13-3. The affinity-purified polyclonal antibody against Munc13-3 (AI 2) recognized a major band of 240 kDa in rat brain and HEK293 cells expressing recombinant Munc13-3, whereas recombinant Munc13-2 or Munc13-3 was not detected.

These novel antibodies were used to study the distribution of Munc13 proteins with a special emphasis on the tissue specificity, regional distribution and subcellular localization. All Munc13 proteins were expressed in a brain-specific manner (Figure 4, upper panel), supporting previously published Northern blot data [7]. The expression pattern of Munc13 proteins reflected the distribution of Munc13 mRNA species as determined by hybridization *in situ* (Figure 2 and Figure 4, lower panel). Munc13-1 was detected in all brain regions tested (Figure 4, lower panel). In contrast, Munc13-2 and Munc13-3 were expressed in complementary patterns in the brain: Munc13-2 was present mainly in the cerebral cortex, hippocampus and striatum, and Munc13-3 was found mainly in the cerebellum (Figure 4, lower panel).

Subcellular fractionation experiments were performed on rat cerebral cortex homogenates to obtain information on the subcellular localization of Munc13 proteins. A general difference between the isoforms was observed with respect to their association with membranes and the cytoskeleton: like Munc13-1, Munc13-3 seemed to be associated mostly with membrane fractions, whereas equal amounts of Munc13-2 were detected in membrane and soluble fractions (Figure 5). We first used a Ficoll

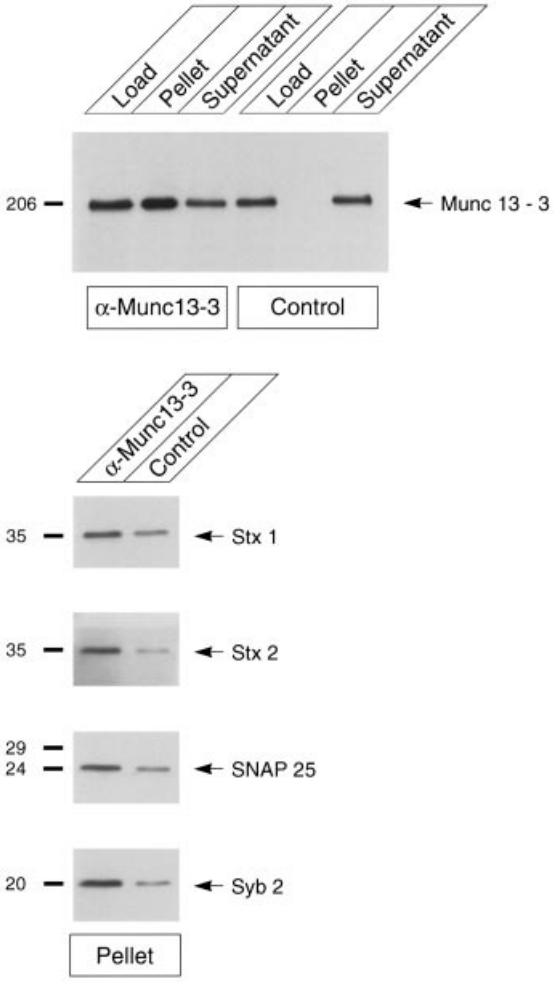

Figure 7 Binding of syntaxin by Munc13-3 in immunoprecipitates

Immunoprecipitations were performed with affinity-purified anti-Munc13-3 polyclonal antibodies (α-Munc13-3) or with preimmune serum (control). Fractions of the extract (load), supernatant after immunoprecipitation, and immunoprecipitate (pellet) were assayed by being immunoblotted with antibodies specific for the indicated proteins. Abbreviations: Stx1/Stx2, syntaxin 1/2; Syb2, synaptobrevin 2. Note the co-precipitation of Munc13-3 with the core complex components syntaxin 1/2, SNAP25 and synaptobrevin 2. Several control proteins were not co-precipitated (NMDA R1, synaptophysin and Munc13-1; results not shown). The molecular masses of protein standards are indicated (in kDa) at the left.

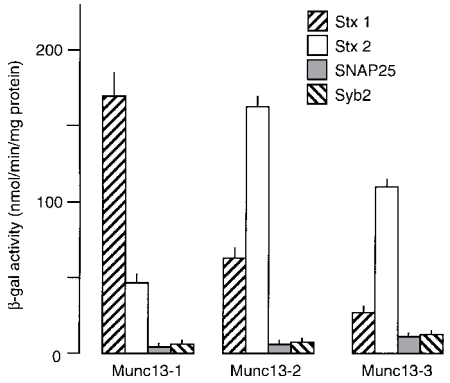

Figure 8 Syntaxin binding by Munc13 isoforms in the yeast two-hybrid system

Yeast two-hybrid interaction assay measuring the β-galactosidase activity of yeast extracts obtained from L40 cultures co-transformed with Munc13 bait constructs and the indicated prey constructs. Note that all Munc13 isoforms bind syntaxins 1B and 2 but not synaptobrevin 2 or SNAP25. Results are means from a representative experiment (triplicate determinations); error bars indicate S.E.M.

gradient centrifugation protocol to prepare relatively pure rat brain synaptosomes. In these experiments, Munc13-1 and Munc13-3 were co-enriched with synaptic markers in the pure synaptosomal fractions (Ficoll Gradient 9/12), indicating a synaptic localization; Munc13-2 was concentrated in the cytosolic fraction (S2; Figure 5A), but a significant fraction of Munc13-2 protein was also detected in purified synaptosomes.

On subcellular fractionation of rat cortex by differential centrifugation, all Munc13 isoforms were enriched in membrane fractions of synaptic origin. Munc13-1 and Munc13-2 were most abundant in synaptic plasma membranes, with a significant fraction of Munc13-2 detectable in the cytosol (Figure 5B). Interestingly, Munc13-3 was strongly enriched in a light membrane fraction containing transport vesicles (P3) and in a crude synaptic vesicle fraction (LP2) (Figures 5B and 5C). It co-migrated with synaptotagmin I on purification of synaptic vesicles by chromatography of LP2 on controlled-pore glass beads (Figure 5C), indicating an association with synaptic or transport vesicles. To analyse the association of Munc13-3 with vesicles in more detail, we performed immunobead precipitations of synaptic vesicles by using anti-synaptophysin antibodies immobilized on microbeads [26]. In contrast with controlled-pore glass bead chromatography, in which the particle dimension determines the separation characteristics, the immunobead procedure allows the isolation of synaptophysin-containing synaptic vesicles of very high purity and their separation from membrane components with similar physical properties but of different protein compositions. We found that Munc13-3 did not co-precipitate with vesicles containing synaptophysin and synaptotagmin I (Figure 5D), suggesting that Munc13-3 resides on a membrane fraction that does not represent synaptic vesicles containing synaptophysin and synaptotagmin I, but one with similar physical properties.

Our biochemical results suggested a synaptic localization and function of Munc13-2 and Munc13-3. To test this further, we examined the developmental expression pattern of the three Munc13 isoforms. Typically, levels of synaptic proteins increase with time after birth. This increase parallels the rate of synaptogenesis and is, for several synaptic vesicle proteins, largely due to changes in post-transcriptional regulation [28]. During development, the onset of expression of Munc13 isoforms coincides with the onset of massive synaptogenesis in the forebrain (Figure 6). None of the three Munc13 proteins was detectable in the embryonic brain (Figure 6, lanes E12 and E16). Munc13 proteins were first detected at birth (Figure 6, lane 0), after which their level of expression was found to increase steadily until it reached a plateau at days 15–22 after birth (lanes P15 and P22).

Interaction of Munc13 proteins with syntaxin

Munc13-1 interacts with syntaxin, an essential component of the synaptic release machinery [9]. We used immunoprecipitation and yeast two-hybrid interaction assays to determine whether binding to syntaxin is a feature common to Munc13 proteins.

For immunoprecipitation experiments, rat brain extracts were incubated with the affinity-purified polyclonal antisera directed against Munc13-2 (AI 1) and Munc13-3 (AI 2). Control experiments were performed with the respective preimmune sera.

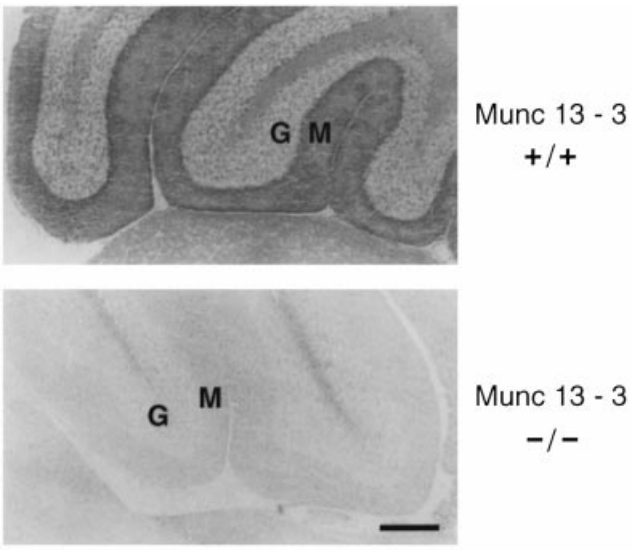

Figure 9 Immunocytochemical localization of Munc13-3

Unfixed cerebellar sections of wild-type (Munc13-3 +/+) mice were stained with an affinity-purified antibody against Munc13-3. Note the strong staining of the molecular layer (M) and sparse labelling of the granule cell layer (G) in wild-type cerebellum. Control sections from Munc13-3 knock-out mice (Munc13-3 −/−) showed only background staining. Scale bar 150 μm.

Immune complexes were precipitated with Protein G–Sepharose and analysed by Western blotting. Although Munc13-2 immunoprecipitations failed owing to poor performance of the antibody (results not shown), Munc13-3 co-precipitated specifically with syntaxin 1 and 2, synaptobrevin 2 and SNAP25 (Figure 7), indicating an association of Munc13-3 with the exocytotic core complex.

As syntaxin binding to Munc13-2 could not be studied in immunoprecipitation assays, we measured the binding of C-terminal Munc13 pLexN bait constructs (residues 1181–1736 of Munc13-1, residues 1463–1985 of Munc13-2, and residues 1702–2204 of Munc13-3, all representing a previously identified, highly homologous syntaxin-binding region [9]) to prey constructs of the three core complex components (syntaxin 1B and syntaxin 2, synaptobrevin, SNAP25 in pVP16-3; same constructs as in [9]) in the yeast two-hybrid system. The two N-terminal syntaxin constructs (syntaxin 1B, residues 1–79, and syntaxin 2, residues 53–227) had previously been identified in a yeast two-hybrid screen with the Munc13-1 C-terminus (residues 1181–1736) and share a conserved coiled-coil domain (H2) that seems to be essential for binding to Munc13-1 [9]. Like Munc13-1, Munc13-2 and Munc13-3 interacted with N-terminal syntaxin constructs (Figure 8). Neither Munc13-1 nor Munc13-2 or Munc13-3 interacted with synaptobrevin or SNAP25 prey constructs (Figure 8).

Taken together, our immunoprecipitation and yeast two-hybrid results indicate that syntaxin binding is a common feature of all Munc13 isoforms and that the interaction of Munc13 proteins with the exocytotic core complex occurs via specific binding to syntaxin.

Immunocytochemical localization of Munc13-3

As all attempts to localize Munc13-2 or Munc13-3 in fixed tissue were unsuccessful, we examined unfixed frozen rat brain for Munc13 immunoreactivity. Immunolabelling of unfixed tissue often leads to non-specific staining and requires stringent control experiments. For that reason we concentrated on immunostaining experiments with Munc13-3 and compared immunolabelling patterns in cerebellar sections obtained from wild-type and Munc13-3 knock-out mice.

Munc13-3 immunolabelling in wild-type cerebellum was homogeneously distributed in the molecular layer. In contrast, the granule cell layer was only moderately labelled around granule cell bodies (Figure 9). No staining of Purkinje cell bodies or Purkinje cell dendrites was apparent, suggesting that the strong labelling in the molecular layer was due to immunopositive presynaptic structures. Control sections obtained from Munc13-3 knock-out mice showed no specific staining. Antibodies against Munc13-2 gave no reliable staining under any of the tested fixation/labelling conditions.

DISCUSSION

Recent direct evidence has demonstrated that rat Munc13-1, a mammalian homologue of *C. elegans unc-13*, is a brain-specific presynaptic protein that serves as a diacylglycerol-dependent, phorbol ester-dependent enhancer of neurotransmitter release [8]. Munc13-1 represents a novel target for the diacylglycerol second messenger pathway that acts in parallel with protein kinase C to modulate presynaptic function [8].

cDNA cloning indicated the presence of at least two additional Munc13 isoforms in rat, Munc13-2 and Munc13-3, but the respective native proteins were never identified [7]. Recombinant Munc13-2 and Munc13-3 proteins have biochemical characteristics that are very similar to those of Munc13-1. They bind phorbol esters and translocate to the plasma membrane in response to the binding of phorbol ester [8]. However, the functional roles of Munc13-2 and Munc13-3 are unknown. The aim of the present study was to identify native Munc13-2 and Munc13-3, to compare their properties with those of Munc13-1, and to determine the functional relationship between the three Munc13 isoforms.

Our results describe a complex set of unique and redundant features of the different Munc13 isoforms. The expression of all Munc13 mRNA species is restricted to neurons. Munc13-1, the most abundant isoform as judged by the number of isolated cDNA species [7], is expressed in all brain regions. In contrast, Munc13-2 mRNA is concentrated in rostral brain regions, whereas Munc13-3 mRNA is found mostly in caudal areas (Figures 2A–2C). Similar patterns of differential and/or complementary mRNA expression were observed within brain regions (hippocampus; Figures 2A–2C) as well as at the cellular level (cerebellar granule and Purkinje cells; Figures 2D–2F). The protein chemical data obtained with isoform-specific antibodies verified the differential distribution of Munc13 isoforms within the rat brain (Figure 4). However, even in brain regions that express very little of the respective mRNA, readily detectable amounts of the corresponding proteins were observed on longer exposure of Western blots (see, for example, Munc13-3 in synaptosomes or subcellular fractions of rat cortex; Figure 5). These findings suggest that each neuronal population (and presumably each neuron) in the rat brain expresses two Munc13 isoforms at high levels, one of which is Munc13-1, whereas the respective third isoform is either absent or present at very low levels.

In a detailed biochemical and immunocytochemical analysis, we attempted to determine the subcellular localizations of Munc13-2 and Munc13-3. We found that the membrane-associated pools of all three Munc13 isoforms are enriched in subcellular fractions of synaptic origin, suggesting a localization to the synapse (Figure 5). These results are in agreement with a

previous immunocytochemical study that demonstrated that Munc13-1 is specifically targeted to presynaptic active zones [8]. They are supported by our immunocytochemical localization of Munc13-3 in wild-type and Munc13-3 knock-out mice (Figure 9). Here, the strong immunolabelling of the cerebellar molecular layer together with the concomitant lack of staining in Purkinje cell bodies and dendrites indicates a preferential staining of granule cell axon terminals and strongly suggests a presynaptic localization of Munc13-3. A synaptic function of Munc13-2 and Munc13-3 is further indicated by the fact that the onset of their expression, like that of Munc13-1, coincides with the onset of massive synaptogenesis in the forebrain (Figure 6). In addition, the finding that all Munc13 proteins interact with syntaxins suggests a presynaptic role for the novel Munc13 isoforms in transmitter release (Figures 7 and 8).

The structural similarity within the Munc13 family as well as between *C. elegans* and mammalian unc-13p-like molecules indicates a functional conservation of these proteins during evolution. Munc13-1 is a presynaptic modulator of neurotransmitter release [8]. In principle, the presence of two additional, closely related isoforms in rat brain might suggest that the different Munc13 proteins (1) have the same (enzymic) function but are not co-localized (e.g. they have similar roles in different brain regions, different cell types or different subcellular compartments), (2) are co-localized but not functionally redundant (e.g. they act as a hetero-oligomeric complex) or (3) are co-localized and functionally redundant.

The finding that the three Munc13 proteins associate differentially with various biochemically purified subcellular fractions (Figure 5) suggests different functional roles for the three Munc13 isoforms in neurons. Munc13-2 is largely soluble, Munc13-1 is associated with synaptic plasma membranes, and Munc13-3 seems to reside on a subpopulation of transport vesicles, suggesting that the three molecules function in different subcellular compartments. However, in view of the striking differential expression patterns of Munc13-1, Munc13-2 and Munc13-3 in rat brain, the significance of differences between the Munc13 proteins with respect to their solubility and subcellular fractionation characteristics is unclear. Indeed, it is unlikely that, for example, Munc13-2 and Munc13-3 perform crucial functions in different subcellular compartments. Their complementary expression patterns demonstrate that a number of brain regions (and therefore neurons) express only one of the two isoforms and can function normally without the other, indicating that Munc13-2 and Munc13-3 perform similar roles in different cells. Given that Munc13-1 is essentially insoluble and is associated with particulate fractions through an interaction with the cytoskeleton [7], it is possible that the differences between the three isoforms in their solubility and subcellular fractionation characteristics are caused by a differential binding to cytoskeletal components.

In contrast with our subcellular fractionation results, several other lines of evidence indicate a partly redundant function of Munc13 isoforms: (1) all Munc13 isoforms are enriched in synaptic fractions (Figure 5), (2) Munc13-1 and Munc13-3 are both targeted to presynaptic terminals [8] (Figure 9), (3) the expression of all Munc13 isoforms coincides with synaptogenesis (Figure 6), (4) all Munc13 isoforms bind syntaxins 1B and 2, suggesting similar functions in transmitter release [9] (Figures 7 and 8), (5) all Munc13 isoforms are receptors for phorbol ester and diacylglycerol [8], (6) Munc13-2 and Munc13-3 have complementary expression patterns, suggesting that the lack of one can be compensated for by the presence of the other (Figures 2 and 4), and (7) Munc13-3 knock-outs are viable and fertile, whereas Munc13-1 knock-outs die after birth (I. Augustin and N. Brose, unpublished work), suggesting a partial functional redundancy of Munc13 isoforms.

On the basis of the present results and preliminary observations in Munc13 deletion mutant mice, we speculate that Munc13-1 has a central role in all neurons of the brain. It might act in concert with, and might replace, either Munc13-2 or Munc13-3, depending on the particular synapse, neuron or brain region. The present results provide a framework for the interpretation of future studies on Munc13 deletion mutations in mice whose phenotypes will allow us to determine more directly the functional role of Munc13 proteins in the nervous system.

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