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A surface antigen delineating a subset of neurons in the primary somatosensory cortex of the mouse

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Abstract. Synapsins are a family of proteins associated with synaptic vesicles that are widely used as markers of synaptic terminals. We decided to investigate synapsin I expression in the mouse primary somatosensory cortex (SI). Immunostaining experiments using a polyclonal antibody against C-terminal domain of synapsin Ia/b (anti-SynI-C) showed an unusual pattern in the SI cortex compared to other regions of the neocortex. The staining delineated the cells located in barrel hollows. The immunoreactive product was located on the perikarya and proximal dendrites of gabaergic neurons found in layer IV and VI of the SI cortex. Other anti-synapsin antibodies did not reveal this pattern within the SI cortex, although in the hippocampus all antibodies examined produced a similar pattern of immunostaining. Deglycosylation of sections resulted in complete loss of immunodecoration on the cell perikarya. We suggest that the anti-SynI-C recognizes a saccharide surface epitope, possibly an element of perineuronal nets that is specific for the primary somatosensory cortex.

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INTRODUCTION

The primary somatosensory cortex (SI) is an area receiving sensory input from mechanoreceptors located on the body surface (Welker 1971). In cortical layer IV of rodents the anatomical representations of body surface are topographically organized and highly segregated. Among them, the barrel cortex contains distinct, ring-like aggregates of neurons (barrels) that form a pattern replicating the distribution of vibrissae on the muzzle (Woolsey and Van der Loos 1970). Barrels in layer IV are the morphological manifestation of functional columns that extend throughout the full thickness of the cortex (Chmielowska et al. 1986, Durham and Woolsey 1985, Kossut and Hand 1984). In mice, each barrel is composed of a densely packed ring of cells (the side) which surrounds a less cell-dense hollow. Adjacent barrels are separated by cell-sparse septa (Rice 1995, Woolsey and Van der Loos 1970).

In adult mice, this characteristic morphological pattern can be mimicked with several postsynaptic proteins such as certain neurotransmitter receptors, e.g. GABA and NMDA receptors, as well as the proteins present in axon terminals, such as glutamic acid decarboxylase, cytochrome oxydase, succinate dehydrogenase, all concentrated in barrel hollows (Czupryn et al. 1998). Lastly, some extracellular matrix proteins, thought to be involved in synapse stabilization also replicate the cytoarchitectonic structure of the barrel cortex (Seo and Geisert 1995, Watanabe et al. 1995).

The synapsins are a family of neuron-specific phosphoproteins associated with synaptic vesicles. The family is composed of five homologous proteins: synapsins Ia and Ib, synapsins IIa and IIb, and synapsin IIIa which are products of alternative splicing of transcripts from three distinct genes (Kao et al. 1998, Sudhof et al. 1989). A comparison of synapsin amino acid sequences reveals a high degree of similarity, especially in the N-terminal domains, which is shared by all isoforms. The C-terminal region shows greater variability (De Camilli et al. 1990, Sudhof 1990). Synapsin I is the most abundant and best characterized of the synapsins and is widely distributed throughout the central nervous system. However, the pattern of its expression is not uniform. In certain regions of the brain there are large differences in the size and shape of immunopositive puncta, labeled by anti-synapsin I antibodies (De Camilli et al. 1983). It has been suggested that this variability reflects differences in the functional properties of synapses (Sudhof 1990).

This study is designed to examine the distribution of synapsin I in the somatosensory cortex of adult mice.

METHODS

Animals, surgical procedures, tissue preparation

The work with animals was carried out in accordance with the European Communities Council Directive (86/609/EEC) and was approved by the Animal Care and Use Committees of the Polish Academy of Sciences. Twenty adult Swiss mice from Nencki Institute animal centre were killed by decapitation, the brains were removed, frozen by immersion in heptane and stored at -70°C, or immediately dissected and homogenized. For immunocytochemistry the frozen brains were then warmed to -20°C and cryostat sections (10 μm) were cut in coronal or tangential planes, mounted onto poly-L-lysine-coated glass microscope slides and stored at -70°C until required. For western blot analysis the dissected cortices were homogenized in ice-cold buffer (1% SDS in PBS) containing protease inhibitor cocktail (Sigma) and the samples were stored at -70°C. Additionally, three mice were deeply anesthetized with Nembutal and perfused transcardially with PBS, pH 7.4 which was followed by a fixative that contained 4% paraformaldehyde. After perfusion the brains were removed and postfixed for 48 h in the same fixative, then frozen and stored at -70°C.

Antibodies and lectins

Synapsin I was detected using goat polyclonal antibody raised against the C-terminal domain of the protein (C-20, sc-8295) diluted 1:1,000. In control experiments goat polyclonal anti-synapsin I antibody raised against the N-terminal domain (anti-SynI-N) was used (N-19, sc-7379) diluted 1:1,000. Both antibodies were obtained from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-synapsin I antibody from Synaptic Systems GmbH was also used (106 001) diluted 1:1,000. Other antibodies were: anti-NeuN (MAB 377) diluted 1:1,000, anti-GAD (AB108) diluted 1:1,000, both obtained from Chemicon and anti-PSD-95 (MA1 046, Affinity Bioreagents, Inc.) diluted 1:1,000. To reveal perineuronal nets we used biotinylated *Wisteria floribunda* agglutinin (L-1766, Sigma) in concentration 5 μg/ml and anti-HNK-1 (347 390, Becton Dickinson), diluted 1:1,000. Secondary biotinylated antibodies

were purchased from Santa Cruz Biotechnology, Inc. and Cy3-conjugated secondary antibodies were obtained from Chemicon.

Immunocytochemistry

The slides were thawed and allowed to dry at room temperature, then fixed for 5 minutes in cold 4% paraformaldehyde (PFA) in PBS. After brief rinses (3 x 5 min) in PBS with 0.4% Triton X-100 (PBS-T), slices were blocked for 1 h at room temperature in a 1.5% solution of normal donkey or normal goat serum (Santa Cruz) diluted in PBS-T. Next, sections were incubated overnight at 4°C with primary antibodies or with N-acetylgalactosamine-specific, biotinylated *Wisteria floribunda* agglutinin (WFA), diluted in the above solution. Following the incubation with respective primary antibody the sections were rinsed in PBS-T (3 x 5 min), and then exposed to either donkey anti-goat or anti-mouse biotinylated IgG (1:200, Santa Cruz) in PBS-T for 30 min at room temperature. After further rinsing in PBS (3 x 5 min) sections were incubated for 30 min with fluorescein-avidin complex (1:25, Vector Laboratories) in bicarbonate-buffered saline solution or with streptavidin-conjugated horseradish peroxidase in PBS. Sections were then mounted in DPX or Vectashield Mounting Medium (Vector). Control experiments included incubation of the sections in the absence of primary or secondary antibodies.

For double immunocytochemistry brain sections were prepared as described above. All incubation times and washing procedures strictly matched those used for single staining. After sections were incubated overnight at 4°C with the first primary antibody, which was usually anti-synapsin, the biotinylated secondary antibody was applied followed by fluorescein-avidin complex. Next, the sections were incubated overnight at 4°C in the second primary antibody and then in the secondary antibody conjugated with Cy3.

Deglycosylation

The deglycosylation procedure was performed as described (Matthews et al. 2002). Briefly, after paraformaldehyde fixation, the sections were subjected to oxidation and deglycosylation. Sections were treated with 10 mM periodate in 50 mM sodium acetate, pH 4.5 for 10 minutes at room temperature, then incubated in 0.3 M sodium borohydride in 0.4 M NaOH for 10 minutes at room temperature. Next, sections were washed 3 times in PBS and further elaborated for immunohistochemistry.

Western blot analysis

Protein samples were separated by electrophoresis on 9% SDS-polyacrylamide gel and electroblotted onto Immobilon-P PVDF membrane (Sigma). Non-specific binding sites were blocked by incubation in 10% milk in TBS for 1.5 h at room temperature. The membrane was then divided into 3 parts and each was incubated with a different anti-synapsin antibody diluted 1:1,000 in TBS with 0.1% Tween-20 (TBS-T). After rinsing 3 times for 5 min in TBS-T the membranes were incubated with biotinylated secondary antibodies in TBS-T for 30 min at room temperature, then washed 3 times with the same buffer and exposed to streptavidin-conjugated horseradish peroxidase for 30 min. After rinsing in TBS-T, protein bands were visualized by exposure of the membranes to DAB in TBS for two minutes.

Image processing

Light microscope images were captured using a computer-assisted camera connected to an Optiphot-2 microscope (Nikon). Some sections were analyzed using a Leica TCS SP2 Spectral Confocal and Multiphoton Microscope. The images were adjusted in brightness and contrast and assembled into panels using Adobe Photoshop 7.0.

RESULTS

Synapsin I protein expression in the neocortex

Previous studies employing immunohistochemical techniques revealed neuropil localization of synapsin I in cortical areas. In this study we confirmed this pattern of synapsin I-like immunostaining. It appeared in virtually all regions of the brain, and consisted of small, diffuse immunoreactive (IR) puncta in neuropil. The puncta were distributed throughout the thickness of the cortex, presumably reflecting the distribution of presynaptic terminals. The most pronounced staining was observed in cortical layer IV (Fig. 1A).

Apart from this dispersed staining another pattern of immunoreactivity was also revealed with the anti-SynI-C antibody. It demarked the shape of some

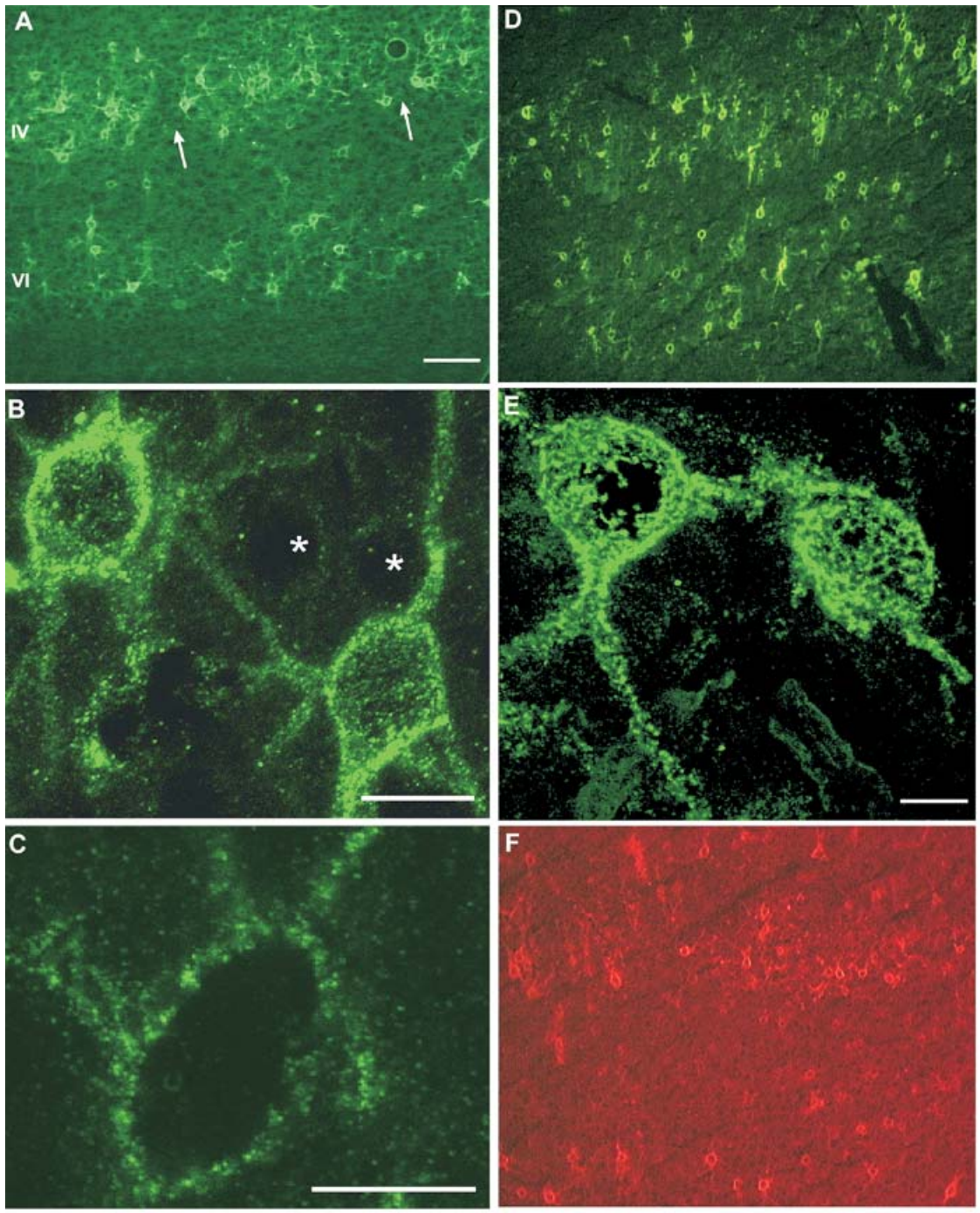

Fig. 1. A coronal section of mouse brain showing the pattern of immunostaining with anti-synapsin C-terminus-specific antibody in the barrel cortex. A, the cells delineated by immunofluorescent product are found mainly in layers IV and VI. Single cells can also be found in layer V. Arrows point to septa between barrels. IV, VI, cortical layers; B, higher magnification of the immunostained neurons in layer IV showing detailed localization of synapsin-IR. The image is a superposition of six confocal sections spaced by 1.2 μm. The immunofluorescent puncta cover the surface of the somata and proximal dendrites of neurons. Asterisks mark non-labeled cellular profiles. Diffuse staining in the neuropil can be observed; C, single confocal image of a neuron located in layer IV. The surface and dendrites are densely covered with immunofluorescent puncta. The interior of the neuron is devoid of staining; D, *Wisteria floribunda*-stained perineuronal nets. The stained profiles are most abundant in layers IV and VI; E, higher magnification of neurons localized in layer IV of somatosensory cortex. Note the lattice-like character of staining; F, HNK-1 labeled cells in the layers IV and VI of the barrel cortex. Scale bar in A, D and F: 100 μm, B and E: 20 μm, C: 8 μm.

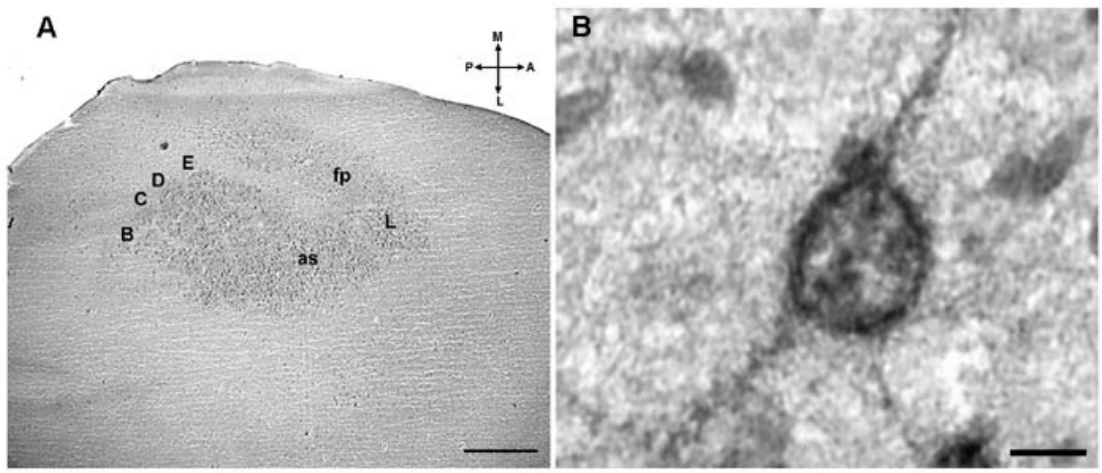

Fig. 2. A tangential section through layer IV of the mouse cortex showing synapsin expression as revealed by the immunoperoxidase method. A, immunoreactivity associated with the somatosensory area. Neighboring regions of the cortex are devoid of cells covered with synapsin immunopositive terminals; B, immunopositive round neuronal profile with few processes located in the barrel hollow. B-E - rows of barrels, as - anterior snout, L - lower lip, fp - forepaw. Scale bar in A: 20 μm, B:1mm. Sections counterstained with cresyl violet.

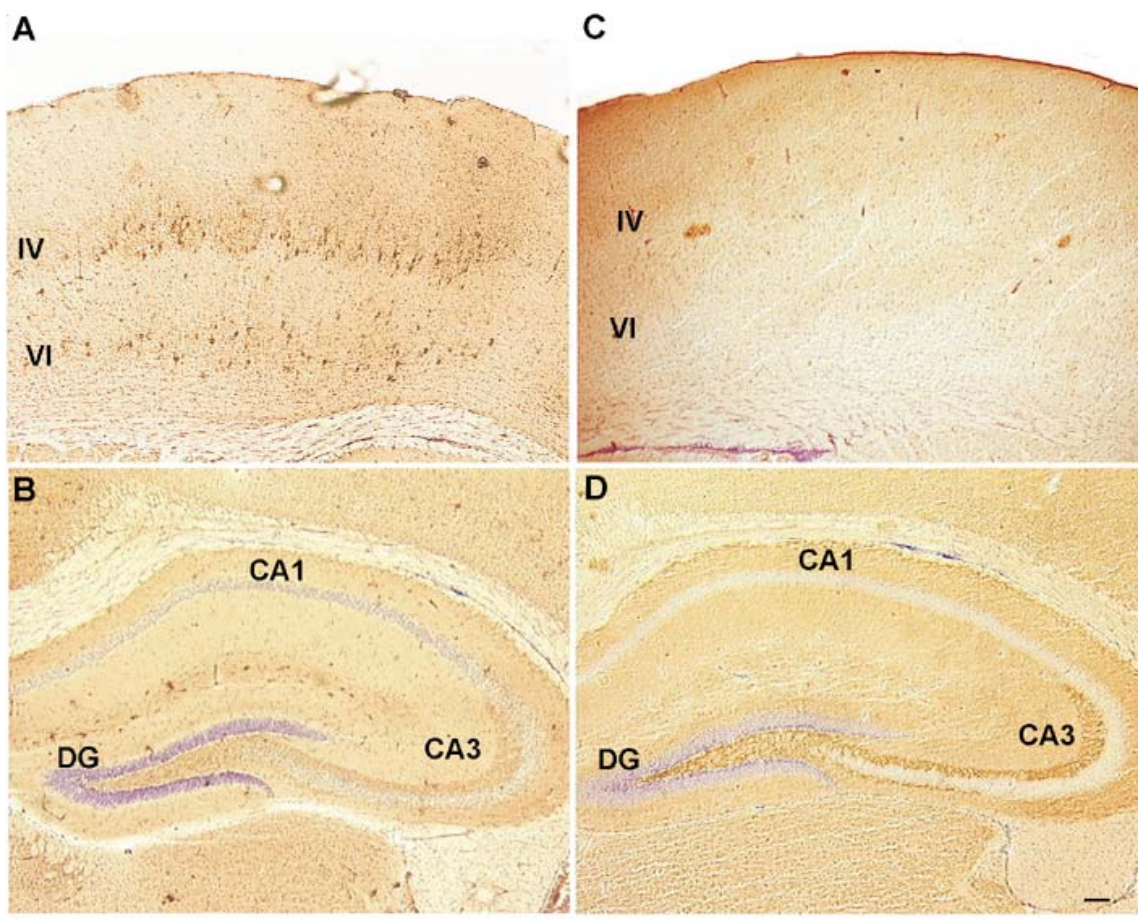

Fig. 3. A comparison of C-terminus (A, B) and N-terminus (C, D) specific anti-synapsin antibodies staining pattern in barrel cortex (A, C) and hippocampus (B, D). A marked difference in the staining pattern produced by the two antibodies can be observed in the somatosensory cortex, whereas in the hippocampus no obvious differences can be seen. IV-VI – cortical layers, CA1, CA3 – fields of hippocampus, DG – dentate gyrus. Immunoperoxidase staining counterstained with cresyl violet. Scale bar: 100 μm.

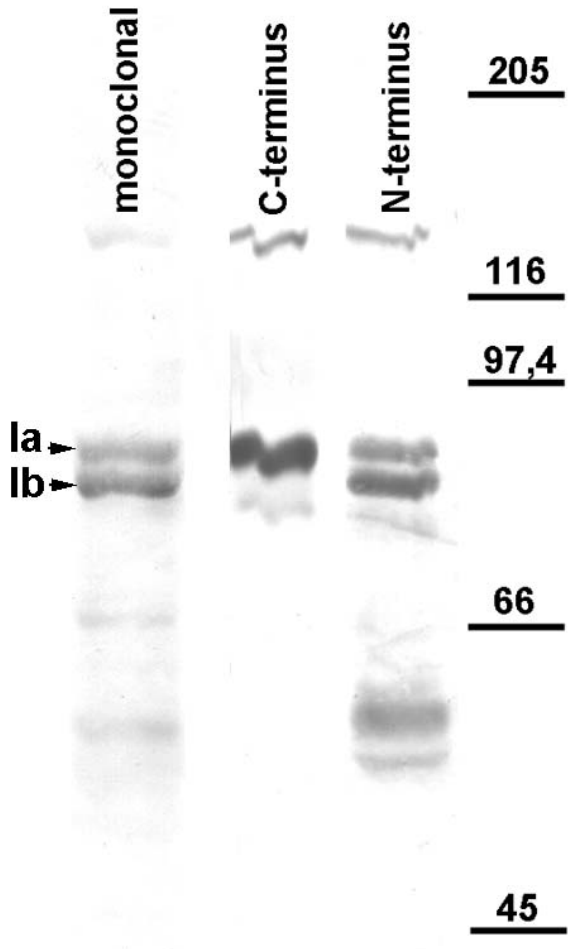

Fig. 4. Western blot analysis of synapsin I isoforms in crude homogenate of the mouse cortex, assesing the specificity of the antibodies used for synapsin I detection. Note that the band corresponding to synapsin Ia is thicker and more intense when detected with the C-terminus-specific antibody as compared to the two other antibodies. Molecular weights in kilodaltons are indicated to the right. Bands corresponding to synapsin Ia and Ib are indicated by arrowheads.

cells present in SI cortex. (Fig. 1A, see also Fig. 3A). Under the confocal microscope it appeared as bright immunoreactive puncta covering the surface of the bodies and proximal parts of the processes of certain cells (Figs.1B and 1C). These cells were observed almost exclusively in layers IV and VI of the barrel cortex and their localization very much resembled the appearance of perineuronal nets as revealed with lectins (*Wisteria floribunda* agglutinin) or monoclonal antibody HNK-1 (Figs. 1D and 1F). The cells were large, (~15-20 μm horizontal diameter), multipolar in shape, with processes arising from the soma in all directions, some of them being slightly biased to the vertical direction (Fig. 1B). Within barrels, somata covered with immunoreactive product were confined to hollows and sides, whereas septa were practically devoid of IR-encrusted cells. The visible parts of the processes did not cross the septa. In layer VI the number of the outlined cells was smaller in comparison to layer IV. Single stained cells were also observed in layer V. When examined at higher magnification, both types of staining distribution, one in the neuropil and the other associated with the soma membrane, appeared conspicuously punctate in character (Fig. 1C).This was in contrast to *Wisteria floribunda* staining, which revealed a lattice-like net covering the surfaces of neurons (Fig. 1E). The immunoreaction performed on tangential sections cut at the level of layer IV revealed that the presence of immunodecorated cells is unique to the primary somatosensory area (SI) of the cortex (Fig. 2A). We observed anti-SynI-C immunoreactive profiles in barrels corresponding to large vibrissae, forepaw pads and to sinus hairs on the lips. In the tangential plane, the cell profiles were round, with fewer processes observed in comparison to the coronal sections (Fig. 2B). The neighboring regions of the neocortex contained only a few single immunodecorated cells.

Specificity of antibody employed

Following rather unexpected pattern of immunoreactivity obtained with anti-SynI-C antibody, we decided to verify it with other anti-synapsin antibodies. To this end we tested Santa Cruz polyclonal antibody against the N-terminus of synapsin I (Fig. 3C), and a monoclonal antibody from Synaptic Systems. Neither of these antibodies immunostained perikaryal membranes or dendrites, despite normal neuropil staining. In the hippocampus however, the pattern of staining was similar for both polyclonal antibodies examined (Figs. 3B and 3D) and a monoclonal antibody (not shown). Staining was found within the neuropil, whereas the surface of neuronal perikarya and dendrites remained unstained. The highest density of immunoreactive product was observed in the hilus and in the CA3 field within the mossy fiber terminal zone. In order to determine the specificity of anti-synapsin antibodies used for immunohistochemistry, we prepared western immunoblots from neocortex homogenates and incu-

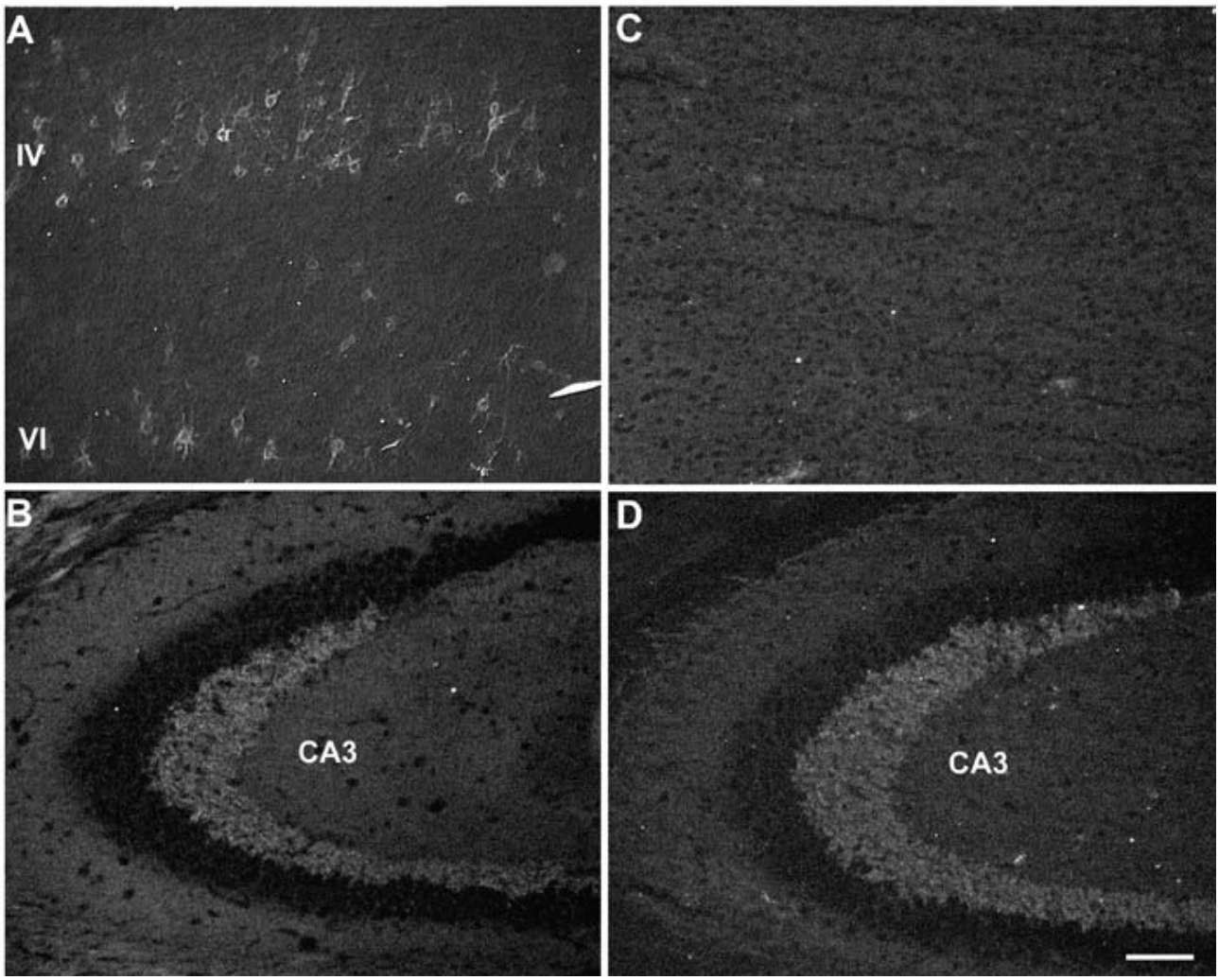

Fig. 5. The effect of chemical deglycosylation on the reactivity of anti-SynI-C antibody. (A, B), untreated sections; A, somatosensory cortex; B, CA3 field of the hippocampus. (C, D), deglycosylated sections; C, somatosensory cortex; D, CA3 field of the hippocampus. Note the disappearance of characteristic immunodecoration of neurons in the cortex (C). Interestingly, the labeling pattern in hippocampus (D) remained unchanged. Scale bar: 100 μm.

bated them with the three various anti-synapsin antibodies. All of the antibodies recognized two major bands at 86 and 80 kDa, corresponding to synapsin Ia and synapsin Ib, respectively (Fig. 4). Interestingly, the band corresponding to synapsin Ia appeared to be much more intense when detected with the anti-SynI-C antibody although the same amount of protein was loaded in all lanes, which might suggest the presence of an additional protein of unknown identity, co-migrating with synapsin Ia isoform. Both monoclonal antibody and anti-SynI-N antibody, recognized additional bands, presumably corresponding to synapsin IIa and IIb, and synapsin IIIa. Thus, the anti-SynI-C antibody used in our experiments appeared to be even more specific as compared to the two other antibodies.

Deglycosylation of sections

To determine the nature of the detected antigen we performed chemical deglycosylation on tissue sections, next subjected to immunohistochemistry. We revealed that this procedure completely abolished the characteristic pattern obtained with the anti-SynI-C antibody and no immunodecorated cells were observed (Fig. 5C). In contrast, deglycosylation did not influence the dispersed component of staining in the cortex. Similarly, in the

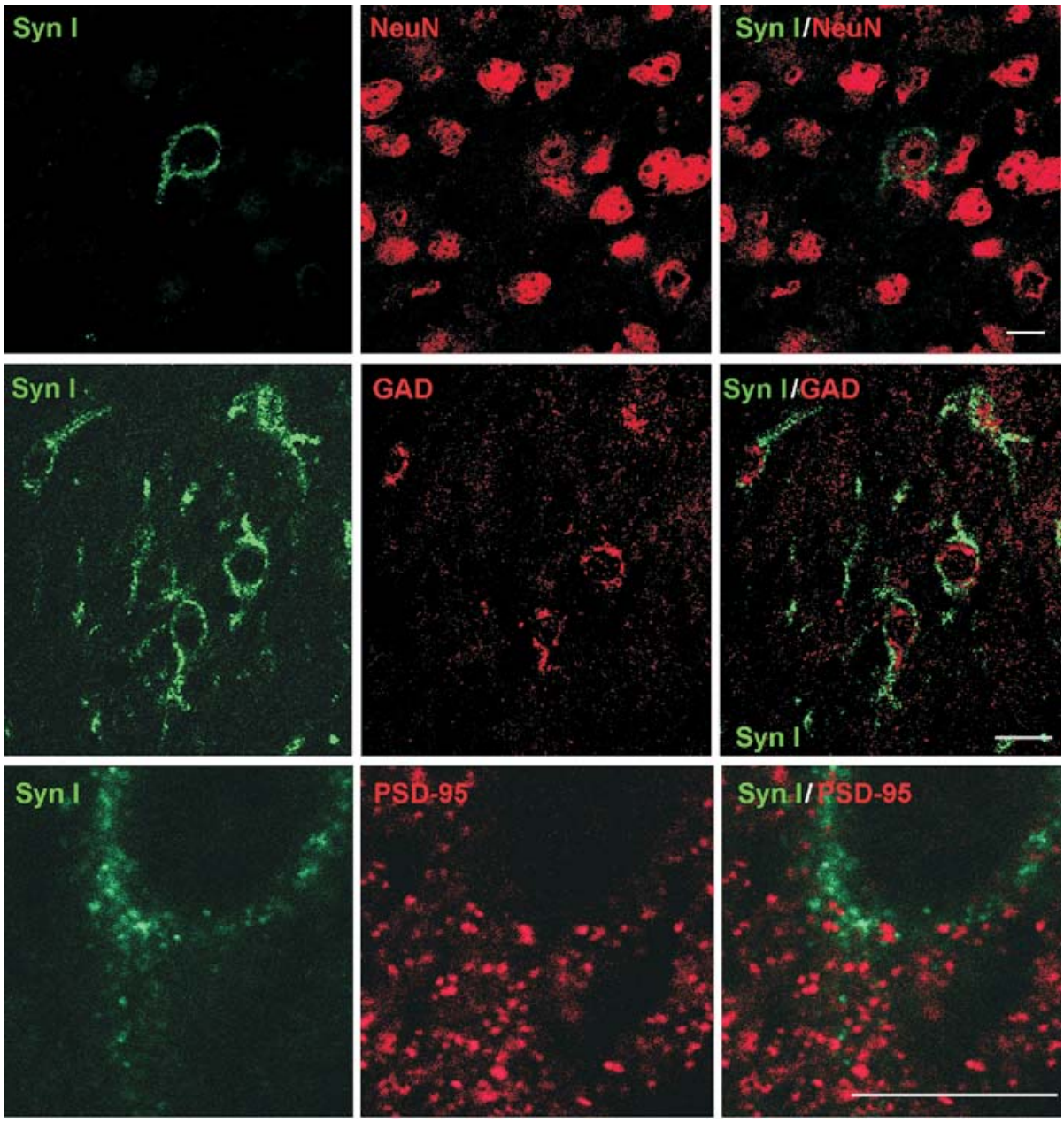

Fig. 6. Confocal images of double immunofluorescent staining for synapsin (green) and NeuN (red) top panel, synapsin (green) and GAD (red) middle panel or synapsin (green) and PSD-95 (red) bottom panel. Superimposed images reveal that all neurons bearing synapsin-like immunopositive puncta on the surface of their somata are GAD-positive. Dense punctate staining for PSD-95 can be seen in the neuropil surrounding non-labeled cellular profiles. Note the virtual lack of co-localization of synapsin and PSD-95 both in puncta covering the neuronal soma and in the neuropil. Scale bar: 10 μm on top and bottom panels, 20 μm on middle panel.

hippocampus no differences between untreated and deglycosylated sections were observed (Figs. 5B and 5D).

Identification of immunodecorated cells

To determine the identity of cells covered with immunopositive puncta we co-localized synapsin I-like labeling and NeuN, a neuron-specific marker. All cells that were covered with IR puncta, were also found to express NeuN, suggesting that they were neurons (Fig. 6, top panel). Interestingly, the staining of the nuclei of these neurons was weaker and punctate, in comparison to surrounding cells that were not covered with synapsin-like immunopositive puncta. To further char-

acterize the type of the neurons we performed double immunostaining for synapsin and glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme. We revealed that most if not all of the cells covered with IR terminals were positive for GAD, suggesting their gabaergic origin (Fig. 6, middle panel). To test the possibility that the immunoreactive puncta are synapses we co-localized synapsin I and PSD-95, a marker of excitatory asymmetric synapses. No co-localization of these two proteins was observed in puncta covering the surface of neuronal somata and dendrites (Fig. 6, bottom panel).

DISCUSSION

In this study, we applied immunocytochemistry with anti-synapsin I antibodies to examine the distribution of synapsin I in the barrel cortex of adult mice. Surprisingly, these antibodies revealed two distinct patterns of immunostaining within SI. The first was dispersed and punctate in character and was present virtually throughout the whole cortical thickness. The second, delineating cell bodies and proximal dendrites of a particular class of gabaergic neurons, was present almost exclusively in layer IV and VI of SI cortex. Furthermore, in layer IV this second type of immunolabeling outlined the barrel field with immunodecorated cells concentrated in the core of barrels that contrasted with empty barrel septa.

The appearance of punctate and disperse immunostaining detected with the anti-SynI-C antibody was in agreement with the distribution of synapsin I revealed by us with anti-SynI-N antibody and monoclonal antibody raised against synapsin I as well as with the localization of synapsin I reported by other authors (De Camilli et al. 1983, Stettler et al. 1996). The second pattern of immunostaining was exclusive for the anti-SynI-C antibody from Santa Cruz and was not found with other anti-synapsin antibodies employed.

The reason of these discrepancies remains elusive. One possibility is that the C-terminus-specific antibody detected preferentially an unknown isoform of synapsin I that is expressed by specific class of neurons present in SI cortex, co-migrating with the synapsin Ia protein on SDS gels. This notion seemed to be strengthened by the result of western blot analysis that revealed that the band corresponding to synapsin I protein was thicker when detected with the anti-SynI-C antibody in comparison to the other antibodies. Another possibility is that this particular antibody detected the modified form of synapsin I. It is known that synapsin I undergoes various posttranslational modifications, namely phosphorylation and glycosylation (Benfenati et al. 1991, Cole and Hart 1999, Greengard et al. 1993, Luthi et al. 1991, Sihra et al. 1989). It is well established that glycosylation sites are localized within the C-terminal part of the protein (Cole and Hart 1999). It is possible that the anti-SynI-C antibody against synapsin detects the glycosylated part of the synapsin molecule with higher affinity than the other antibodies. This possibility is strongly supported by the experiment showing the disappearance of IR-profiles following the deglycosylation procedure.

Another explanation is that this antibody, apart from synapsin I, reacted with an unidentified surface antigen expressed by a particular class of neurons. The pattern of the immunoreaction is strikingly similar to that revealed by the markers of perineuronal nets (Hartig et al. 1992). A perineuronal net is a specialized form of extracellular matrix covering the surface of a subset of neurons, often of gabaergic type (Celio and Blumcke 1994, Kosaka et al. 1989, Kosaka and Heizmann 1989). It contains mainly proteoglycans bearing large polysaccharide chains on their protein cores. A comparison of *Wisteria floribunda* agglutinin-stained neurons with those observed by us revealed similarities in the localization of staining. In both cases the neuronal somata and their proximal dendrites were outlined by an immunofluorescent product. Some differences, however, could be observed. The staining with *Wisteria floribunda* agglutinin produced a lattice-like net covering the surface of neurons, whereas the character of staining with the anti-SynI-C antibody was rather punctate. Another difference is that the *Wisteria* agglutinin decorated neuron in many cortical areas (Bruckner et al. 1994), whereas the staining with anti-SynI-C antibody was confined to the primary somatosensory cortex.

The possibility that the anti-SynI-C antibody detects a certain element of the perineuronal nets seems to be likely, especially in view of the deglycosylation experiments. After deglycosylation of the tissue sections the immunodecorated neurons became invisible. Simultaneously, the punctate, dispersed staining in the cortex remained unchanged and the pattern of immunostaining in the hippocampus was well preserved. These results strongly suggest that the epitope recognized by the antibody is localized on the carbohydrate part of the mole-

cule. Furthermore, it appeared that immunodecoreated neurons could be preferentially visualized in unfixed tissue and the application of perfusion with 4% paraformaldehyde seemed to abolish this peculiar immunostaining. The nature of this epitope and its detailed localization needs further investigation. Perineuronal nets are extremely heterogenous in their molecular composition, varying both in their protein structure and the carbohydrate side chains (Celio and Blumcke 1994). Several different antibodies or lectins recognizing different epitopes localized on perineuronal nets were described (Celio and Blumcke 1994). As confirmed by us, *Wisteria floribunda* agglutinin staining reveals cells located mainly in layers IV and VI, in virtually all cortical areas including hippocampus, as well as in some subcortical structures (Bruckner et al. 1994). Monoclonal antibodies of Cat-3xx family recognizing various forms of large chondroitin sulfate proteoglycan, aggrecan, also visualize perineuronal nets and recognize epitopes on distinct, although overlapping neuronal subpopulations (Lander et al. 1998, Matthews et al. 2002). Cat-301 antibody strongly labels neurons in the brainstem and spinal cord of the rat, few in other areas. In a marked contrast, Cat-315 and Cat-316 antibodies mark neurons in the forebrain but only few in the brainstem and spinal cord (Lander et al. 1998); significant interspecies differences in labeling have been described (Celio and Blumcke 1994). Another monoclonal antibody, HNK-1, raised against a carbohydrate epitope containing a sulfated derivative of glucuronic acid stains selectively a population of gabaergic neurons in the parietal cortex of the rat (Ren et al. 1994). Some cells in other areas, such as cingular cortex and hippocampus, cerebellum and spinal cord are also recognized by this antibody (Ren et al. 1994, Rollenhagen et al. 2001). The expression of this epitope very much resembles the pattern of expression obtained by us with the anti-SynI-C antibody, although in the cerebrum the latter seemed to be even more restricted to the primary somatosensory area.

CONCLUSIONS

In the present study we show that the C-terminus-specific anti-synapsin antibody selectively recognizes an unidentified surface antigen delineating a subset of gabaergic neurons in the primary somatosensory area of the mouse. We reveal that the expression pattern obtained with this antibody is similar but not identical to HNK-1. The antigen revealed by this antibody exhibits cell type specificity and may lend to certain neurons molecular surface identity. We suggest that the epitope revealed by us may represent a useful tool for delineating primary somatosensory cortex.

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ABBREVIATIONS

Anti-SynI-C	-	C-terminus-specific anti-synapsin I antibody
Anti-SynI-N	-	N-terminus-specific anti-synapsin I antibody
GABA	-	gamma aminobutyric acid
GAD	-	glutamic acid decarboxylase
IR	-	immunoreactivity
LTP	-	long term potentiation
PBS	-	phosphate buffered saline
PBS-T	-	phosphate buffered saline - Triton X-100
PMSF	-	Phenylmethylsulfonylfluoride
PSD-95	-	postsynaptic density protein 95
PVDF	-	polyvinylidene difluoride
SI	-	primary somatosensory cortex
SSC	-	standard saline citrate
TBS	-	Tris buffered saline
TBS-T	-	Tris buffered saline – Tween 20
WFA	-	*Wisteria floribunda* agglutinin

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