The Polysialylated Form of the Neural Cell Adhesion Molecule (PSA-NCAM) Is Expressed in a Subpopulation of Mature Cortical Interneurons Characterized by Reduced Structural Features and Connectivity

María Angeles Gómez-Climent1, Ramón Guirado1, Esther Castillo-Gómez1, Emilio Varea1, María Gutierrez-Mecinas1,5, Javier Gilabert-Juan1,2, Clara García-Mompó1, Sandra Videira1, David Sanchez-Mataredona1, Samuel Hernández1, José Miguel Blasco-Ibañez1, Carlos Crespo1, Urs Rutishauser3, Melitta Schachner4 and Juan Nacher1

1Neurobiology Unit and Program in Basic and Applied Neurosciences, Department of Cell Biology, Universitat de València, 46100 Burjassot, Spain, 2Centro de Investigación Biomédica en Red de Salud Mental, 3Laboratory of Developmental Neuroscience, Cell Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA, 4Zentrum fuer Molekuleare Neurobiologie, Universitaetsklinikum Eppendorf, D-20246 Hamburg, Germany and 5Current address: Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Bristol BS1 3NY, UK

Gómez-Climent and Guirado have contributed equally to this work

Address correspondence to Dr Juan Nacher, Neurobiology Unit, Department of Cell Biology, Universitat de València, Dr. Moliner, 50, 46100 Burjassot, Spain. Email: nacher@uv.es.

Principal neurons in the adult cerebral cortex undergo synaptic, dendritic, and spine remodeling in response to different stimuli, and several reports have demonstrated that the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) participates in these plastic processes. However, there is only limited information on the expression of this molecule on interneurons and on its role in the structural plasticity of these cells. We have found that PSA-NCAM is expressed in mature interneurons widely distributed in all the extension of the cerebral cortex and have excluded the expression of this molecule in most principal cells. Although PSA-NCAM expression is generally considered a marker of immature neurons, birth-dating analyses reveal that these interneurons do not have an adult or perinatal origin and that they are generated during embryonic development. PSA-NCAM expressing interneurons show reduced density of perisomatic and peridendritic puncta expressing different synaptic markers and receive less perisomatic synapses, when compared with interneurons lacking this molecule. Moreover, they have reduced dendritic arborization and spine density. These data indicate that PSA-NCAM expression is important for the connectivity of interneurons in the adult cerebral cortex and that its regulation may play an important role in the structural plasticity of inhibitory networks.

Keywords: dendrite remodeling, dendritic arborization, interneuron subtypes, NCAM, neuronal structural plasticity, spine remodeling

Introduction

The neural cell adhesion molecule (NCAM), through the action of 2 polysialyltransferases, ST8SiaII and ST8SiaIV (Hildebrandt et al. 2008), is able to incorporate long chains of polysialic acid (PSA), which confers it antiadhesive properties. Consequently, PSA-NCAM expression facilitates structural remodeling and is involved in several neurodevelopmental events, such as neuronal migration, neurite outgrowth, or synaptogenesis (for review, see Bonfanti 2006; Gascon et al. 2007; Rutishauser 2008). Although NCAM is the major carrier of PSA in the central nervous system (CNS) (Hildebrandt et al. 2008), this sugar has also been detected on other proteins, such as neuropilin2 (Curreli et al. 2007), a sodium channel subunit (Zuber et al. 1992), SynCAM1 (Galuska et al. 2010), and the polysialyltransferases St8SiaII and St8SiaIV (Muhlenhoff et al. 1996; Close and Colley 1998).

PSA-NCAM is very abundant during CNS development, but it can also be found in several regions and cell populations during adulthood. Some of these cells are immature neurons, such as those found in the adult neurogenic regions (Seki and Arai 1993b; Rousselot et al. 1995; Bonfanti 2006) and the paleocortex layer II (Gomez-Climent et al. 2008). In addition, previous studies have indicated that PSA is also present in a subpopulation of mature interneurons in the hippocampus and the medial prefrontal cortex (mPFC) (Nacher, Blasco-Ibanez, and McEwen 2002; Varea et al. 2005; Varea, Castillo-Gomez, et al. 2007b). Moreover, pharmacological modulation of serotonergic or dopaminergic neurotransmission results in parallel changes in the expression of PSA and molecules involved in γ-aminobutyric acidergic (GABAergic) neurotransmission in the mPFC of adult rats (Saaranen et al. 2007; Varea, Blasco-Ibanez, et al. 2007; Varea, Castillo-Gomez, et al. 2007b; Castillo-Gomez et al. 2008). Altogether, these results suggest that, at least in the mPFC, changes in PSA expression may be involved in the plasticity of inhibitory circuits. However, we still do not know whether similar interneurons express PSA in other cortical regions or whether this molecule is exclusively expressed by a subpopulation of interneurons and absent from most principal cells. Moreover, the functional significance of PSA expression in interneurons is still unresolved. The study of interneurons expressing PSA is also interesting because, although there is considerable information on the structural plasticity of cortical principal neurons, few reports have addressed this subject in inhibitory neurons (Lee et al. 2006, 2008).

The aims of this study are: 1) to offer a complete mapping of PSA expressing cells in the adult rodent cerebral cortex, 2) to determine the neurochemical phenotype of somata and neuropil elements expressing PSA in the adult cerebral cortex and to exclude its expression in principal cells, 3) to demonstrate that NCAM is the only carrier of PSA in adult cortical interneurons, using NCAM knockout mice, 4) to demonstrate that PSA expression in interneurons is not affected by genetic deletion of NCAM in principal cells, 5) to study the time of origin of PSA expressing interneurons and to exclude their postnatal generation, 6) to study the presence/density of different types of...
synapses on PSA expressing interneurons and their ultrastructure and to compare them with those of interneurons lacking this molecule, and 7) by using transgenic mice expressing green fluorescent protein (GFP) under the promoter of GAD67, to analyze the dendritic arborization and spine density of PSA expressing interneurons, and to compare it with that of interneurons lacking PSA expression.

Materials and Methods

Animals and Treatments

Rats
Forty-one young adult male Sprague-Dawley (SD) rats (3 months old, 300 ± 15 g, Harlan Iberica) were separated into different groups. 1) Eight rats were used to study the neurochemical phenotype of PSA expressing cells and their perisomatic and peridendritic puncta, using immunohistochemistry and confocal microscopy. The phenotype of PSA expressing puncta in the neuropil was also analyzed in these animals. 2) Thirty rats were used to study whether PSA immunoreactive cells were generated during adulthood, using double PSA/5′BrdU immunohistochemistry. All the rats in this group received 4 intraperitoneal (i.p.) injections, each 12 h. of 5′BrdU (Sigma; 50 mg/kg, in sterile saline) when they were 3 months old and were sacrificed 2, 4, 7, 14, 21, or 30 days after the last injection (n = 4 per group). 3) Three SD rats were used for PSA preembedding immunohistochemistry for electron microscopy.

Twelve perinatal SD male rats were used to study whether PSA immunoreactive cells were generated during perinatal development, using double PSA/5′BrdU immunohistochemistry. The rats in this group underwent the same 5′BrdU injection protocol described above at postnatal days P0, P10, and P20 (n = 4 per group) and were sacrificed when they were 3 months old.

Eight pregnant SD rats (Harlan Iberica) received 2 i.p. injections of 5′BrdU (50 mg/kg), 8 h apart, on the following days after coupling: E11.5, E13.5, E15.5, and E17.5 (n = 2 per group) in order to identify whether PSA expressing cells were generated during embryogenesis. The first 24 h after coupling were designated as embryonic day 0 (E0). Four males (3 months old, 306 ± 32 g) were selected from each offspring and processed for double PSA/5′BrdU immunohistochemistry.

Mice
Twelve young adult male GIN (GFP-expressing inhibitory neurons, Tg(GadGFP)+570+Swm) mice (3 months old, 27.5 ± 4 g, purchased from Jackson laboratories and bred in our animal facility) were separated into different groups. 1) Six mice were used to study the neurochemical phenotype of GFP/PSA-expressing interneurons using confocal microscopy and immunohistochemistry for several markers. 2) Six mice were used for the analysis of dendritic arborization and spine density of GFP-expressing interneurons with or without PSA expression.

Three B6.Cg-Tg(Thy1-YFP)2Jrs/J young adult male mice (3 months old, 268 ± 5 g, purchased from Jackson laboratories and bred in our animal facility), in which subsets of pyramidal neurons are "Golgi-like" labeled (Gogolla et al. 2009), were used to exclude the expression of PSA in pyramidal neurons of the cerebral cortex.

Five NCAM−/− mice and 5 wild-type littermates on a C57BL/6 background (males, 3 months old) (Cremer et al. 1994) were used to study whether PSA expression in interneurons was associated to NCAM expression. 10 wild-type littermates on a C57BL/6 background (males, 3 months old) were used to study whether PSA expression was present in principal neurons. These mice were generated by a Cre-loxP recombination system to generate a mutant, in which the NCAM gene is ablated under the control of the CaMKII promoter (Bukalo et al. 2004). These animals were processed for double PSA/GAD67 immunofluorescence as described below.

Histological Procedures
Rats and mice destined for confocal microscopy studies were perfused transcardially under deep chloral hydrate anesthesia, with saline and then 4% paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4 (PB). Rat brains destined for fluorescence immunohistochemistry were cut with a vibratome (Leica VT 1000E, Leica), and 50-μm thick coronal sections were collected and kept in cold PB (4 °C) before processing. In the case of GIN mice, the brains were cut into 150-μm thick sections with a vibratome for the study of dendritic arborization and dendritic spine density. Brains for conventional immunohistochemistry were cryoprotected with 30% sucrose in PB, coronal sections (50 μm) were obtained with a sliding freezing microtome (Leica SM2000R) and stored at -20 °C in 30% glycerol, 30% ethylene glycol in PB until used.

The rats processed for electron microscopy were perfused transcardially under deep chloral hydrate anesthesia, first with saline for 1 min, followed by 450-mL solution of paraformaldehyde 2% in a lysine-phosphate buffer, pH 7.4 (see Supplementary experimental procedures). Brains were then extracted and sliced with a vibratome at 50 μm as described above.

All animal experimentation was conducted in accordance with the European Communities Council Directive of November 24th 1986 (86/609/EEC) and was approved by the Committee on Bioethics of the Universitat de València. Every effort was made to minimize the number of animals used and their suffering.

Immunohistochemistry for Conventional Light Microscopy, Fluorescence Microscopy, and 5′BrdU Detection

Tissue was processed 'free-floating' for immunohistochemistry as described (Nacher et al. 2005; Varea et al. 2005). Detailed information on the methodology and the specificity of the anti-PSA antibody can be found in the Supplementary experimental procedures section. This antibody recognizes exclusively the PSA (Rougon et al. 1986), but since in the adult CNS most, if not all, PSA expression is associated to NCAM, it is frequently denominated anti-PSA-NCAM (Rutishauser 2008).

In order to characterize neurochemically the somata and neurite elements expressing PSA as well as the immunoreactive puncta apposed to the PSA-expressing somata and dendrites, we have performed double or triple immunohistochemistry using primary antibodies against PSA and against different markers of immature neurons, mature neurons, interneurons, astrocytes, oligodendrocytes, microglia, proteins related to neuronal activity, and synaptic contacts (see Supplementary Table 1). For detailed information on PSA/5′BrdU immunohistochemistry, see Supplementary experimental procedures section.

Observation and Quantification of Double-Labeled PSA-Expressing Somata

All sections processed for fluorescence immunohistochemistry were mounted on slides and coverslipped using DakoCytomation fluorescence mounting medium (Dako North America, Inc.). Then, the sections were observed under a confocal microscope (Leica TCS SPE) using a ×63 oil objective. Z-series of optical sections (1-μm apart) were obtained using the sequential scanning mode. These stacks were processed with LSM 5 image software. One in 10 series of telencephalic sections from each animal was double-labeled as described. Fifty randomly selected immunoreactive cells were analyzed in each case to determine the coexpression of PSA and the markers described above. This study was performed in deep layers of the cortex layer III and lateral entorhinal cortex layers III to VI, in the nongranule cells of hippocampus and in the mPFC, in order to complete previous studies (Nacher, Blasco-Ibanez, and McEwen 2002; Varea et al. 2005). A similar methodology was applied when analyzing PSA expression in pyramidal neurons of Thy1-YFP mice. Seventy pyramidal neurons (selected by morphological criteria) per animal were analyzed in all the extension of the neocortex and paleocortex.
Quantification of PSA-Expressing Somata and Neuronal Immunoreactivity in the Hippocampus of NCAMff- and NCAMff-Transgenic Mice

The number of PSA-expressing neurons in the hippocampal CA1 region was estimated using a modified version of the fractionator method (West 1993), as described before (Nacher, Alonso-Llosa, et al. 2002). In order to determine PSA immunoreactivity intensity, tissue was analyzed with confocal microscopy in order to obtain optical densities of the regions of interest. A detailed description of the stereological and densitometric procedures can be found in the Supplementary experimental procedures section. Means were determined for NCAMff+ and NCAMff- mice, and the data were subjected to statistical analysis using the unpaired Student’s t-test.

Analysis of Perisomatic and Peridendritic Puncta on PSA-Expressing Interneurons

All sections processed for fluorescence immunohistochemistry were processed as described above and observed under a confocal microscope. Z-series of optical sections (0.5-μm apart) were obtained using sequential scanning mode. Twenty randomly selected PSA-expressing cells, from deep layers of paleocortex and nongranule cells of hippocampus in each case, were analyzed using different presynaptic and postsynaptic markers (Table 1). The profile of these cells was drawn and only puncta contacting this profile were analyzed. The localization of PSA and synaptic markers was analyzed in 4 consecutive confocal planes of each selected neuron, in which the penetration of both antibodies was optimal.

In order to compare the number of perisomatic and peridendritic puncta between PSA-expressing interneurons and those lacking PSA expression, we analyzed a randomly selected sample of glutamic acid decarboxylase-67 (GAD67)-expressing cells of the CA3 region in the hippocampus in each case, were analyzed using different presynaptic and postsynaptic markers (Table 1). The profile of these cells was drawn and only puncta contacting this profile were analyzed. The localization of PSA and synaptic markers was analyzed in 4 consecutive confocal planes of each selected neuron, in which the penetration of both antibodies was optimal.

The number of perisomatic and peridendritic puncta that coexpress the different cellular markers. GFAP, glial fibrillary acidic protein.

Table 1 Phenotypic characterization of PSA-expressing cells in the rat Paleocortex deep layers, mPFC, and in hippocampal nongranule neurons

<table>
<thead>
<tr>
<th>Markers</th>
<th>Paleocortex deep layer</th>
<th>Hippocampal nongranule cells</th>
<th>Prefrontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td>0</td>
<td>52 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>c-FOS</td>
<td>10 ± 4</td>
<td>2 ± 0.5</td>
<td>6 ± 0.5</td>
</tr>
<tr>
<td>CAMKII</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CB</td>
<td>30 ± 8</td>
<td>10 ± 2</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>CCK</td>
<td>17 ± 5</td>
<td>54 ± 10</td>
<td>16 ± 0</td>
</tr>
<tr>
<td>CR</td>
<td>0</td>
<td>33 ± 7</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>GAD67</td>
<td>77 ± 6.9</td>
<td>84 ± 1</td>
<td>59 ± 7</td>
</tr>
<tr>
<td>GFAP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GR</td>
<td>47 ± 1</td>
<td>8 ± 0.5</td>
<td>90 ± 1.5</td>
</tr>
<tr>
<td>NeuN</td>
<td>83 ± 4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NPY</td>
<td>23.3 ± 3.5</td>
<td>9 ± 4</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>NR1</td>
<td>97 ± 1</td>
<td>91 ± 1</td>
<td>91 ± 1</td>
</tr>
<tr>
<td>OX-42</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-CREB</td>
<td>20 ± 2</td>
<td>34 ± 6</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>PV</td>
<td>20 ± 5</td>
<td>6 ± 2</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Rip</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SST</td>
<td>53 ± 7.6</td>
<td>8 ± 0.5</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>VIP</td>
<td>2 ± 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S’Blud-E 11.5</td>
<td>10 ± 2</td>
<td>9 ± 1.4</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>S’Blud-E 13.5</td>
<td>25 ± 1</td>
<td>18 ± 1</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>S’Blud-E 15.5</td>
<td>35 ± 5</td>
<td>39 ± 11</td>
<td>25 ± 3.5</td>
</tr>
<tr>
<td>S’Blud-E 17.5</td>
<td>12.25 ± 4.34</td>
<td>5.44 ± 1.28</td>
<td>15.76 ± 4.83</td>
</tr>
</tbody>
</table>

Percentages of GAD67 expressing cells co-expressing PSA in the rat cerebral cortex

<table>
<thead>
<tr>
<th>Markers</th>
<th>Paleocortex</th>
<th>Hippocampus</th>
<th>Prefrontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>10.5 ± 1.5</td>
<td>9 ± 2</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

Note: Numbers indicate the percentage (=standard error of the mean) of PSA-expressing cells that coexpress the different cellular markers. GFAP, glial fibrillary acidic protein.

Analysis of PSA-Expressing Puncta in the Neuronal

All sections were processed and observed as described above. Z-series of optical sections (0.5-μm apart) were obtained using sequential scanning mode. One hundred PSA immunoreactive puncta were analyzed in each region and animal (n = 4) to determine the coexpression of PSA and different markers of excitatory and inhibitory elements and general synaptic markers (Table 1). This study was performed in deep layers of entorhinal and prelimbic cortices.

Aridin-Biotin-Immunoperoxidase and Immunogold-Silver Detection for Electron Microscopy

The sections destined to electron microscopy were cryoprotected for 30 min by immersion in 25% sucrose and 10% glycerol in 0.01 M PB and then underwent freeze thawing 3 times with liquid nitrogen to enhance antibody penetration. Detailed information on the methodology used for preembedding avidin-biotin-immunoperoxidase and preembedding immunogold-silver detection for electron microscopy, as well as on the inclusion and ultramicrotomy, can be found in the Supplementary experimental procedures section.

Analysis of Synaptic Density in the Perisomatic Region of PSA Versus Non-PSA-Expressing Interneurons. Ultrastructural Analysis of PSA Expression in Pyramidal Neuron Somata

The density of synaptic contacts on the plasma membrane was studied analyzing profiles of interneuron somata from selected regions of the ventral hippocampus and the deep layers of mPFC, where PSA interneurons were specially abundant, both from DAB- and gold-stained material. The interneurons were identified by their profound invagination of the nucleus and an electron-dense cytoplasm with high density of mitochondria, endoplasmic reticulum, and Golgi apparatus. Nine interneurons expressing PSA in the plasma membrane and 9 lacking expression of this molecule were analyzed in each region.

Thirty profiles of pyramidal neuron somata of the mPFC cortex were also studied under the electron microscope in order to analyze PSA expression in their perisomatic region.

Analysis of Dendritic Arborization and Spine Density in GAD-GFP and PSA/GAD-GFP Expressing Interneurons

A detailed description of the stereological and densitometric procedures can be found in the Supplementary procedures section.

Results

**PSA-Expressing Cells with Typical Interneuron Morphology Are Widely Distributed in the Adult Rodent Telencephalon**

The distribution of PSA-expressing cells was similar in the rat and mouse telencephalon (Fig. 1). PSA-expressing cells, mostly multiform and resembling typical interneurons, were found widely dispersed in every region and layer of the neocortex, although they were more abundant in deep layers. These cells also populated the deep layers of paleocortex (piriform cortex layer III and entorhinal cortex layers III to VI) but were extremely rare in layers II or I. In layer II, most of the cells expressing PSA were tangled or semilunar-pyramidal transitional neurons (Gomez-Climent et al. 2008). The distribution of PSA-expressing nongranule neurons in the hippocampus was similar to that described previously in the rat (Nacher, Blasco-Ibanez, and McEwen 2002).

PSA expression was detected in the neopil of neocortical and paleocortical regions and was generally more intense in deep layers, as described before (Nacher, Alonso-Llosa, et al.
Hippocampal PSA expression was in consonance with previous reports (Seki and Aral 1993). In some cases, particularly in neocortical layers III and V, some PSA-expressing puncta were not colocalized with the marker of putative astrocytes (Nacher, Alonso-Llosa, et al. 2002; Varea et al. 2005; Varea, Castillo-Gomez, et al. 2007). 

In order to know whether the expression of PSA interferes with the activity of interneurons, we studied the expression of the most commonly used cell activity markers in the CNS, c-Fos, and Arc. Only very low percentages of the PSA-expressing interneurons studied coexpressed c-Fos (Table 1). The percentages of Arc coexpression were also low, except in the case of hippocampal nongranule cells (Table 1 and Supplementary Fig. 5A). By contrast, most PSA-expressing cells in all the regions studied coexpressed the NR1 NMDA receptor subunit (Table 1). The expression of glucocorticoid receptor (GRs) in PSA-expressing cells was variable among the different areas studied (Table 1): Most of these cells coexpressed GR in the mPFC, about half of them coexpressed this receptor in the paleocortex, but only very few hippocampal nongranule cells coexpressed PSA and GR (Table 1 and Supplementary Fig. 5B).

None of the PSA-expressing puncta in the neuropil of deep layers of entorhinal and prelimbic cortices expressed the vesicular glutamate transporter 1 (VGAT, Fig. 3A), which is associated with synaptic vesicles in GABAergic synapses and is crucial for inhibitory function (McIntire et al. 1997). GAD67 was also expressed, with similar percentages in some PSA-expressing puncta (Fig. 3B and Table 2), except in the neuropil of the prelimbic cortex, where a higher degree of colocalization was observed. In agreement with the VGAT and GAD67 coexpression, we also found that a similar percentage of PSA-expressing puncta in every region studied was closely apposed to puncta-expressing gephyrin (Fig. 3D and Table 2), a protein involved in the clustering of glycine and GAB(A) receptors, which is detectable in the postsynaptic density of inhibitory synapses (Fritschy et al. 2008). A small percentage of

PSA-Expressing Elements in the Cortical Neuropil Correspond to Inhibitory Structures

None of the PSA-expressing puncta in the neuropil of deep layers of entorhinal and prelimbic cortices expressed the vesicular glutamate transporter 1 (VGLUT1, Fig. 3A), a molecule responsible for the active transport of l-glutamate into synaptic vesicles, which is generally used as a marker of excitatory synapses in the telencephalon.

By contrast, we found that, in all the regions studied, some PSA-expressing puncta coexpressed the vesicular GABA transporter (VGAT, Fig. 3B and Table 2), which is associated with synaptic vesicles in GABAergic synapses and is crucial for inhibitory function (McIntire et al. 1997). GAD67 was also expressed, with similar percentages in some PSA-expressing puncta (Fig. 3B and Table 2), except in the neuropil of the prelimbic cortex, where a higher degree of colocalization was observed. In agreement with the VGAT and GAD67 coexpression, we also found that a similar percentage of PSA-expressing puncta in every region studied was closely opposed to puncta-expressing gephyrin (Fig. 3D and Table 2), a protein involved in the clustering of glycine and GAB(A) receptors, which is detectable in the postsynaptic density of inhibitory synapses (Fritschy et al. 2008). A small percentage of

PSA-Expressing Interneurons Express Low Levels of Cell Activity Markers but High Levels of NMDA and Glucocorticoid Receptors

In order to know whether the expression of PSA interferes with the activity of interneurons, we studied the expression of the most commonly used cell activity markers in the CNS, c-Fos, and Arc. Only very low percentages of the PSA-expressing interneurons studied coexpressed c-Fos (Table 1). The percentages of Arc coexpression were also low, except in the case of hippocampal nongranule cells (Table 1 and Supplementary Fig. 5A). By contrast, most PSA-expressing cells in all the regions studied coexpressed the NR1 NMDA receptor subunit (Table 1). The expression of glucocorticoid receptor (GRs) in PSA-expressing cells was variable among the different areas studied (Table 1): Most of these cells coexpressed GR in the mPFC, about half of them coexpressed this receptor in the paleocortex, but only very few hippocampal nongranule cells coexpressed PSA and GR (Table 1 and Supplementary Fig. 5B).

PSA-Expressing Interneurons Have Reduced Dendritic Arborization and Spine Density Compared With Interneurons Lacking PSA-NCAM

The somata of PSA-immunoreactive cells with the characteristic morphology of interneurons have been represented as small dots. PSA-immunoreactive cells in the hippocampal subgranular zone and the paleocortex layer II have been omitted from the drawings. Scale bar: 3.4 mm for A and 2 mm for B. The profiles of the representative sections have been modified from those in Paxinos and Watson (1986) and Paxinos and Franklin (1997).
PSA-expressing puncta also coexpressed SYN, a synaptic vesicle membrane protein (Greengard et al. 1993), in the neuropil of all the areas analyzed (Fig. 3E and Table 2).

**PSA-Expressing Interneurons Are Generated during Embryonic Development**

*Embryonic Neurogenesis*

The analysis of the hippocampus, paleocortex, and mPFC of adult rats, which received 5′BrdU at different embryonic stages (E11.5, E13.5, E15.5, and E17.5), revealed that the highest proportion of PSA-expressing cells displaying 5′BrdU-labeled nuclei was found in those injected at E15.5 (Supplementary Fig. 5C), although there were small differences between the 3 regions analyzed (for details, see Table 1).

*Early Postnatal and Adult Neurogenesis*

In all the groups, some scarce 5′BrdU-labeled nuclei were found in all the regions studied (many of them appeared in pairs), we never observed any of these nuclei located inside a PSA-expressing soma. By contrast, several PSA-immunoreactive cells displaying a 5′BrdU-labeled nucleus could be observed in areas with known...
adult neurogenic activity (subventricular zone, rostral migratory stream, olfactory bulb, and subgranular zone). This expression pattern was observed in 1) the animals injected with 5'BrdU in postnatal days P0, P10, and P20 and sacrificed when 3 months old and 2) in the 3-month-old rats injected with 5'BrdU and sacrificed 2, 4, 7, 14, 21, or 30 days later.

**NCAM Is the Only Polysialylated Protein in Interneurons**

Although NCAM is the major carrier of PSA in the vertebrate CNS (Hildebrandt et al. 2008), different reports have described the presence of this complex sugar in some other proteins (for details, see Introduction). Since the antibody used in this study recognizes only PSA but not the protein to which it is attached,
we analyzed inhibitory elements in NCAM-deficient mice using PSA immunohistochemistry. Our study revealed the absence of PSA expression in cortical somata or neuropil elements expressing GAD67 (Fig. 4A,B), indicating that in inhibitory elements, PSA was exclusively associated to NCAM. Consequently, from this point, the antigen recognized by our antibody is denominated PSA-NCAM.

The Expression of PSA-NCAM in Interneurons Is Not Affected by Genetic Deletion of NCAM in Principal Cells

The analysis of PSA-NCAM expression in NCAMf+ mice (in which the NCAM gene is ablated under the control of the CaMKII promoter and thus absent from principal neurons) has revealed very few differences when comparing these animals with their wild-type littermates. NCAMf+ mice lacked PSA-NCAM expression in their hippocampal mossy fibers but presented several labeled cells in the SGZ of the dentate gyrus, as described (Bukalo et al. 2004). Typical PSA-NCAM-expressing interneurons were found in all the regions studied, with similar morphology and distribution to those described in GIN mice, many of which coexpressed GAD67 (Fig. 4C,D). We estimated the number of PSA-NCAM-expressing cells in the CA1 region of the entire hippocampus, and there were no significant differences between NCAMf− (1892 ± 224.4) and NCAMf+ mice (2004 ± 70.8) (Unpaired student’s t-test, P = 0.86). However, densitometric analysis revealed that NCAMf+ mice showed a small, but significant, reduction in PSA-NCAM expression in the neuropil of CA1 lacunosum molecule (wild type: 83,556 ± 7,601, NCAMf+: 28,667 ± 3,908; P = 0.046, Fig. 4E,F). This reduction was not detectable in deep layers of the infralimbic cortex (wild type: 24,443 ± 6,738, NCAMf+: 36,113 ± 10,794; P = 0.932, Fig. 4G,H).

PSA-NCAM-Expressing Interneurons Have Lower Density of Perisomatic and Peridendritic Puncta than Interneurons Lacking PSA-NCAM

The analysis of perisomatic and peridendritic puncta on PSA-NCAM-expressing interneurons was performed in deep layers of the paleocortex and in the ventral hippocampus (Tables 3 and 4). These cells displayed perisomatic and peridendritic puncta expressing different markers of presynaptic excitatory (VGLUT1, Fig. 5A) and inhibitory (VGAT and GAD67, Fig. 5B,C) elements as well as the general synaptic marker SYN (Fig. 6A).

The differences in the number of SYN-expressing puncta per micron of soma or dendrite profile between interneurons expressing PSA-NCAM with those lacking this molecule were studied in the ventral hippocampus (Table 4, Fig. 6). In this region, the number of puncta per micron was significantly lower in PSA-NCAM-expressing interneurons, both in the perisomatic and in the peridendritic regions (unpaired Student’s t-test, P = 0.0005 and P = 0.001, respectively).

PSA-NCAM-Expressing Interneurons Have Lower Density of Perisomatic Synapses than Interneurons Lacking PSA-NCAM

The ultrastructure of PSA-NCAM-expressing nongranule neurons in the ventral region of the hippocampus and of interneurons in deep layers of the mPFC was studied with transmission electron microscopy (Fig. 7). All the cells studied showed typical interneuron features, such as a profound invagination of the nucleus, reduced soma size when compared with pyramidal neurons, and an electron-dense cytoplasm with high density of mitochondria, endoplasmic reticulum, and Golgi apparatus (Schwartzkroin and Kunkel 1985; Babb et al. 1988). Excitatory and inhibitory synaptic contacts were observed in the perisomatic region of these interneurons. PSA-NCAM expression was found restricted to the plasma membrane and located exclusively at the extracellular surface (Fig. 7A,B). Although immunolabeling was present in most of the surface of the somata studied, it was never found on the surface contacting a presynaptic membrane. By contrast, pyramidal neuron somata in the hippocampus and mPFC cortex never showed PSA-NCAM expression in their plasma membrane.

Since the qualitative estimation of the number of perisomatic synaptic contacts per micron of membrane in these hippocampal PSA-NCAM interneurons revealed differences with those lacking PSA-NCAM expression in their vicinity, a detailed comparison of this number was made. The number of perisomatic synaptic contacts normalized to the cell perimeters sampled in PSA-NCAM-expressing interneurons was significantly smaller than in those lacking expression of this molecule (Fig. 7C).

PSA-NCAM-Expressing Interneurons Have Reduced Dendritic Arborization and Spine Density Compared With Interneurons Lacking PSA-NCAM

In the ventral hippocampus, Sholl analysis revealed reduced dendritic arborization in GFP+/PSA-NCAM+/expressing interneurons when compared with GFP+/PSA-NCAM− cells (Fig. 8A,B). These differences were significant in the 2–6 segments (20 µm) of distance from the soma, reaching a maximum difference in the fourth segment (Fig. 8C). The number of bifurcations was also significantly smaller in PSA-NCAM-expressing interneurons (Fig. 8D).

The analysis of dendritic spine density also showed significant differences in the dendrites of the PSA-NCAM-expressing

### Table 2

<table>
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<tr>
<th>Markers</th>
<th>Paleocortex</th>
<th>Prelimbic cortex</th>
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<tbody>
<tr>
<td>VGAT</td>
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<td>15 ± 2</td>
</tr>
<tr>
<td>GAD67</td>
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<td>15 ± 1</td>
</tr>
<tr>
<td>Gephyrin</td>
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<td>15 ± 0.5</td>
</tr>
<tr>
<td>SYN</td>
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<td>15 ± 0.5</td>
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### Table 3

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<th>Entorhinal cortex (soma)</th>
<th>Entorhinal cortex (dendrites)</th>
<th>Nongranule cells (soma)</th>
<th>Nongranule cells (dendrites)</th>
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</thead>
<tbody>
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<td>GAD67</td>
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<td>SYN</td>
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<td>0.33 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.24 ± 0.1</td>
</tr>
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### Table 4

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<tr>
<th>Markers</th>
<th>Nongranule cells (perisomatic puncta)</th>
<th>Nongranule cells (peridendritic puncta)</th>
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<tr>
<td>GAD67+</td>
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<td>0.66 ± 0.08</td>
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<tr>
<td>GAD67−</td>
<td>0.19 ± 0.03</td>
<td>0.24 ± 0.04</td>
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interneurons (Fig. 8E,F). The dendrites of interneurons expressing GFP were divided into 3 segments of 60 μm. We found a significant reduction in the dendritic spine density of PSA-NCAM-expressing interneurons in the 2 distal segments (Fig. 8G). This reduction was also significant when taking into account the whole length of the dendrite fragment studied (Fig. 8H).

**Discussion**

**PSA-NCAM-Expressing Cells Are Mature Interneurons Widely Distributed in the Adult Rodent Cerebral Cortex**

The present results describe a widespread distribution of mature PSA-expressing neurons in the adult rodent cerebral cortex. They expand previous findings describing the existence of PSA-expressing cells in the hippocampus outside the subgranular zone (Nacher, Blasco-Ibanez, and McEwen 2002) and in the mPFC (Varea, Castillo-Gomez, et al. 2007). Other authors previously also acknowledged briefly the existence of these cells in the hippocampus (Bonfanti et al. 1992) and in the ventral cingulate cortex of rodents (Seki and Arai 1991) and the neocortex, hippocampal formation, and entorhinal cortex of humans (Mikkonen et al. 1999; Arellano et al. 2002; Varea, Castillo-Gomez, et al. 2007). However, a detailed analysis of the distribution of these cells and their phenotype has only been performed in the hippocampus and mPFC of rodents.

Although NCAM is not the exclusive carrier of PSA (Zuber et al. 1992; Close and Colley 1998; Curreli et al. 2007; Galuska et al. 2010), the present results strongly suggest that NCAM is its only carrier in cortical interneurons. Consequently, following the terminology used in our previous reports and in that of other laboratories, in this discussion and the appropriate parts of the Results section, we refer to this molecule as PSA-NCAM.

Due to its intense expression in immature neurons, PSA-NCAM has been erroneously considered in many studies an exclusive developmental marker. However, previous works have clearly demonstrated its presence in mature neurons and its involvement in their structural plasticity (for review, see...
Bonfanti 2006; Gascon et al. 2007; Rutishauser 2008). The present work also argues against this incorrect view and clearly indicates that most PSA-NCAM—expressing cells outside adult neurogenic regions and paleocortex layer II are mature neurons, most of which have been generated during embryonic development.

Different lines of evidence indicate that mature cortical neurons expressing PSA-NCAM constitute a subpopulation of interneurons: 1) the expression of the enzyme responsible for GABA synthesis (revealed by GAD67 immunohistochemistry and by the analysis of transgenic mice expressing GFP under the GAD67 promoter), 2) the ultrastructural characteristics, and 3) the expression of different neuropeptides and calcium-binding proteins considered exclusive markers of mature interneurons.

The fact that many PSA-NCAM—expressing cells do not appear to colocalize with inhibitory markers can be explained because none of these markers is present in all interneurons. Even the expression of GABA or their synthesizing enzymes is not found in all interneuronal somata; some interneurons, depending on their activity or the distance to their axonal terminals, may have low levels of these molecules in their somata (Freund and Buzsaki 1996). The differences observed in the percentages of PSA-NCAM/GAD67—expressing hippocampal nongranule neurons between rats and mice may be due to species differences in the expression of the enzyme or in its antigenicity since the methodology used and the region studied were the same in both species.

PSA-NCAM—expressing elements in the cortical neuropil also appear to belong to inhibitory neurons because they express markers associated with interneuronal neurites or synapses. Most of these elements must correspond to neurites of local interneurons, although, since PSA-NCAM is expressed in interneurons of extracortical regions, such as the amygdala (Nacher J and Gomez-Climent MA, unpublished data) or the septum (Foley et al. 2003), some of them may correspond to projections from extracortical origin.

Moreover, our data demonstrate that PSA-NCAM expression is clearly absent from mature principal neuron somata in the adult cerebral cortex, using electron microscopy, CAMKII

Figure 5. Confocal microscopic analysis of perisomatic and peridendritic puncta on PSA-NCAM—expressing interneurons of adult rats. Ventral hippocampus. (A) VGLUT1—expressing puncta on PSA-NCAM—expressing cells. (B and C) VGAT (B) and GAD67 (C) expressing puncta on PSA-NCAM—expressing cells. Note the abundance of perisomatic and peridendritic puncta on the cell surface (insets). All the images in this figure are taken from single confocal planes. Scale bar: 5 μm. (Insets in the figures are ×2 enlargements.) GAD67, glutamate decarboxylase, isoform 67.
immunohistochemistry, and transgenic mice, in which pyramidal neurons can be unequivocally identified by YFP expression. The absence of PSA-NCAM expression in principal neurons of the neocortex is also supported by our analysis of NCAM++ transgenic mice, in which NCAM is absent from excitatory neurons. However, certain mature excitatory neuronal populations in the hippocampus of wild-type rodents express PSA-NCAM only in their axons or their terminal boutons: the axons (mossy fibers) of most mature granule cells in the hilus and CA3 stratum lucidum (Seki and Arai 1999) and the terminal boutons of axons of CA3 pyramidal neurons (Schaffer collaterals), some of which terminate in the CA1 stratum lacunosum moleculare (Muller et al. 1996; Schuster et al. 2001). This may explain the lack of PSA-NCAM expression in the mossy fibers of NCAM++ mice (Bukalo et al. 2004), and its reduction in the stratum lacunosum moleculare (present results). However, in NCAM++ mice, not only PSA but also NCAM are ablated and, consequently, the effects observed may be NCAM dependent.

Cortical interneurons can be classified on basis of their neurochemical properties by the expression of different calcium-binding proteins and neuropeptides (for review, see Freund and Buzsaki 1996; Markram et al. 2004; Ascoli et al. 2008). The present results indicate that cortical PSA-NCAM-expressing interneurons do not belong to any of the previously described categories. Moreover, there are substantial differences in the coexpression of PSA-NCAM and different calcium-binding proteins/neuropeptides between different cortical regions, especially in the hippocampus. In the paleocortex and neocortex, many of the PSA-NCAM-expressing cells express calbindin and somatostatin, while few of them express parvalbumin, calretinin, neuropeptide Y, or vasointestinal peptide. By contrast, in the hippocampus, many PSA-NCAM-expressing nongranule neurons express calretinin. It is also conceivable that some interneurons do not express PSA-NCAM in their somata but only in their terminal fields.

Possible Dynamics of PSA-NCAM Expression in Interneurons

In contrast to PSA-NCAM expression in the hippocampal subgranular zone or the paleocortex layer II, which is strongly downregulated during aging (Seki and Arai 1995; Abrous et al. 1997; Varea et al. 2009), PSA-NCAM expression in cortical interneurons is stable over lifetime (both in the number of PSA-NCAM-expressing cells and in the intensity of PSA-NCAM-expressing neuropil) (Varea et al. 2009). However, we do not know whether PSA-NCAM is expressed always in the same subgroup of interneurons or whether different groups of interneurons can switch on and off this expression depending on synaptic activity. It is also possible that all, or a subset, of PSA-NCAM-expressing interneurons retain this expression constitutively since their generation during embryonic development. In any case, our laboratory has demonstrated that different pharmacological manipulations can increase or decrease the number of PSA-NCAM-expressing neurons in the

Figure 6. Confocal microscopic analysis of perisomatic and peridendritic puncta on PSA-NCAM-expressing versus non-PSA-NCAM-expressing interneurons of adult rats. Ventral hippocampus. (A) SYN-expressing puncta on a cell coexpressing PSA-NCAM and GAD67. (B) SYN-expressing puncta on a GAD67-expressing cell lacking PSA-NCAM expression. All the images in this figure are taken from single confocal planes. Scale bar: 5 μm. (Insets in the figures are ×2 enlargements.) GAD67, glutamate decarboxylase, isoform 67.
indicating that, at least to some extent, inhibitory neurons can stop expressing, express de novo, or reexpress PSA-NCAM. Although there are reports on postnatal generation of interneurons in the neocortex (Cameron and Dayer 2008; Ohira et al. 2010), our BrdU studies do not support the possibility that PSA-NCAM-expressing interneurons belong to this subpopulation of recently generated neurons.

Functional Significance of PSA-NCAM Expression in Interneurons

The most important issue in our study is the question relating to the physiological consequences for an interneuron to express PSA-NCAM on its plasma membrane. Two nonexcluding hypotheses can be formulated. First, the presence of PSA-NCAM reduces cell-to-cell and cell-to-extracellular matrix adhesion, thus allowing interneurons to remodel the structure of their neurites, spines, and/or synapses. Thus, the morphology of PSA-NCAM-expressing interneurons may be just an instantaneous "picture" of a dynamic remodeling process. Whether PSA-NCAM expression is necessary for arbor rearrangements observed in cortical interneurons in vivo (Lee et al. 2006) remains to be explored. Logically, the next step would be to study the effects of PSA depletion on interneuronal structure and preliminary experiments in our laboratory indicate that these effects are indeed intense.

The second hypothesis would imply that PSA-NCAM expression plays an insulatory role, restricting the possibility of establishing synaptic contacts in the plasma membrane regions where it is present. In fact, under the electron microscope, we never have found a synapse in a portion of surface displaying PSA-NCAM expression, and previous reports have suggested that reduced NCAM function (for instance by the addition of PSA) may lead to a decreased stability of synaptic contacts (De Paola et al. 2003). We cannot exclude, however, that the absence of PSA-NCAM in some of these synapses may be due to problems with antibody penetration or immunodetection protocols in the material destined to electron microscopic studies. Additionally, it has to be noted that the detection of perisomatic puncta using immunohistochemistry and confocal microscopy suggests, but does not demonstrate, the presence of synapses. Only transmission electron microscopy can clearly show these structures. The spatial resolution provided by conventional confocal microscopy may be insufficient to discriminate the location of the molecules in the synapse or to clearly distinguish the puncta that make synapses with PSA-NCAM–expressing neurons from closely located ones that may not have synaptic contacts.

We have recently described that immature PSA-NCAM–expressing neurons in the adult paleocortex layer II are completely isolated from synaptic input, probably due to the presence of PSA-NCAM and glial processes in most of their surface (Gomez-Climent et al. 2008). By contrast, this insulation is incomplete in PSA-NCAM–expressing interneurons, since we have found that some excitatory and inhibitory puncta are found close to their perisomatic and periendritic surface, and both excitatory and inhibitory synapses contact at least the perisomatic region of these interneurons. Consequently, PSA-NCAM–expressing interneurons are integrated in the cortical circuits but, since they receive less input, they may...
Figure 8. Dendritic arborization and spine density in GFP+/PSA-NCAM− versus GFP+/PSA-NCAM+ expressing interneurons in the ventral hippocampus of GIN mice. (A and B) 3D reconstructions of GFP-expressing interneurons lacking PSA-NCAM expression (A) and coexpressing PSA-NCAM (B). Insets are views of the somata displayed in A and B showing coexpression of PSA-NCAM (up) and GFP (bottom). (C and D) Sholl analysis of GFP-expressing interneurons, showing intersection number per 20-μm dendritic radial unit distance from the soma (C) and bifurcation number (D). White circles (C) and bars (D) indicate interneurons lacking PSA-NCAM expression and black circles and bars correspond to PSA-NCAM-expressing interneurons. (E and F) Compositions, using fragments of different confocal planes, of spinous dendrites of GFP-expressing cells in interneurons expressing PSA-NCAM (E) and in interneurons lacking this molecule (F). (G and H) Histograms of the differences in dendritic spine density in segments at different distances from the soma (G) and the total density of dendritic spines (H). Scale bar = 10 μm (E and F) and 50 μm (A and B). Asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01, ***P < 0.001). Values represent means ± standard error of the mean.
have reduced activity, possibly resulting in reduced dendritic arborization, decreased spine density, and low expression of cell activity markers. Electrophysiology experiments need to be performed to verify this hypothesis. However, new techniques for identifying PSA-NCAM-expressing cells, which do not affect PSA function/structure, should be developed before conducting these experiments. It is tempting to think that PSA-NCAM-expressing interneurons may constitute a reservoir, which, after downregulating PSA-NCAM expression, may receive more synaptic contacts and thus become more integrated in the circuitry. Conversely, PSA-NCAM upregulation in interneurons may lead to the opposite effect.

It has to be noted that, in contrast with our results, a previous report described that PSA-NCAM-expressing neurons receive more synapses than those lacking NCAM (and consequently PSA) (Dityatev et al., 2000), suggesting that the presence of PSA-NCAM may promote synapse formation. However, these were cultured neurons from very young animals. It is possible that in the environment of the control adult cerebral cortex, in which plasticity is reduced, the presence of PSA-NCAM in interneurons may mainly have an insulating role.

The presence of PSA-NCAM on interneurons may also interfere with NCAM-signaling pathways in these cells or their synaptic input. Different reports have demonstrated that polysialylation of NCAM decreases homophilic binding and may influence the interactions with heterophilic-binding partners, such as growth factor receptors (for review, see Gascon et al., 2007) or, as it has been recently described, glutamate receptors (Kochlamazashvili et al., 2010).

In conclusion, expression of PSA-NCAM on interneurons may have important implications on the structure and physiology of cortical inhibitory circuits. This expression may also be relevant for the understanding of the etiology of certain psychiatric disorders, in which alteration of inhibitory networks has been described. Recent work in our laboratory has demonstrated PSA-NCAM expression in the neocortex is regulated by dopamine (Castillo-Gomez et al., 2008) and serotonin (Varea, Blasco-Ibanez, et al., 2007; Varea, Castillo-Gomez, et al., 2007), which are profoundly implicated in the etiology and pharmacological treatment of schizophrenia and major depression. Moreover, this expression is also regulated in animal models of depression, (Pham et al.; 2003; Cordero et al., 2005), and in the hippocampus of schizophrenic patients (Barbeau et al., 1995).

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Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

Notes
Conflict of Interest: None declared.

References


