Chronic Fluoxetine Treatment Increases the Expression of PSA-NCAM in the Medial Prefrontal Cortex

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Recent hypotheses suggest that changes in neuronal structure and connectivity may underlie the etiology of depression. The medial prefrontal cortex (mPFC) is affected by depression and shows neuronal remodeling during adulthood. This plasticity may be mediated by the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), which is intensely expressed in the adult mPFC. As the expression of PSA-NCAM is increased by serotonin in other cerebral regions, antidepressants acting on serotonin reuptake may influence PSA-NCAM expression and thus counteract the effects of depression by modulating neuronal structural plasticity. Using immunohistochemistry, we have studied the relationship between serotonergic fibers and PSA-NCAM expressing neurons in the adult rat mPFC and the expression of serotonin receptors in these cells. The effects of fluoxetine treatment for 14 days on mPFC PSA-NCAM expression have also been analyzed. Although serotonergic fibers usually do not contact PSA-NCAM immunoreactive neurons, most of these cells express 5-HT₃ receptors. In general, chronic fluoxetine treatment induces significant increases in the number of PSA-NCAM immunoreactive neurons and in neuropil immunostaining and coadministration of the 5-HT₃ antagonist ondansetron blocks the effects of fluoxetine on PSA-NCAM expression. These results indicate that fluoxetine, acting through 5-HT₃ receptors, can modulate PSA-NCAM expression in the mPFC. This modulation may mediate the structural plasticity of this cortical region and opens new perspectives on the study of the molecular bases of depression.

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INTRODUCTION

The cellular bases of depression are not yet well understood, although recent hypotheses suggest that it may be caused by dysfunction of the normal mechanisms underlying the plasticity of neuronal networks (Castren, 2005; Duman *et al*, 1999). In fact, antidepressant drugs may act by normalizing this impairment (Duman *et al*, 1999; Manji *et al*, 2000, 2001; Manji and Duman, 2001). Structural plastic processes, such as dendritic or spine remodeling, have been observed in several regions of the adult CNS, specially as a response to aversive experiences (McEwen, 2000), or as a consequence of several learning paradigms (Muller *et al*, 2002). Interestingly, some of the regions displaying this plasticity are those affected by depression, such as the amygdala, the hippocampus, the basal ganglia, and several regions of the medial

prefrontal cortex (mPFC) (Bearden *et al*, 2001; Drevets *et al*, 1997; Fava and Kendler, 2000; Strakowski *et al*, 2000).

Brain imaging studies have demonstrated a reduction in blood flow, volume, number of glial cells, and neurons in the prefrontal cortex of patients suffering depression (Drevets *et al*, 1997; Ongur *et al*, 1998; Rajkowska *et al*, 1999). Neurons in the rodent mPFC undergo changes in their structure under the influence of stress or corticosterone (Radley *et al*, 2004; Wellman, 2001), psychoactive drugs (Robinson and Kolb, 1999), gonadal hormones (Forgie and Kolb, 2003), or changes in blood pressure (Vega *et al*, 2004). This neuronal remodeling in the mPFC may be mediated by changes in the expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), as it appears to occur in regions such as the hippocampus or the amygdala (Cordero *et al*, 2005; Pham *et al*, 2003). NCAM can incorporate long chains of PSA, which confer it antiadhesive properties (Rutishauser, 1996). Consequently, the expression of PSA-NCAM allows neurons to participate in plastic events such as neurite outgrowth (Zhang *et al*, 1992) or synaptic reorganization (Seki and Rutishauser, 1998). Recently, our laboratory has shown the presence of PSA-NCAM in certain neurons and the neuropil of the adult rat mPFC (Varea *et al*, 2005), suggesting that PSA-NCAM may also mediate the structural changes occurring in this region.

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Pyramidal neurons in the mPFC undergo dendritic remodeling after specific prefrontocortical serotonin (5-HT) depletion (Perez-Vega *et al*, 2000), indicating that neuronal structural plasticity in this cortical region may be mediated by serotonergic transmission. General 5-HT depletion also reduces PSA-NCAM expression in the hippocampus, as well as in hypothalamic nuclei and basal ganglia (Brezun and Daszuta, 1999; Brezun and Daszuta, 2000), which indicates a relationship between 5-HT and PSA-NCAM expression. The mPFC receives a dense serotonergic innervation (Azmitia and Segal, 1978; Steinbusch, 1981) and contains several 5-HT receptor subtypes (Pazos *et al*, 1985; Pazos and Palacios, 1985; Pompeiano *et al*, 1992). Consequently, this cortical region is a good target for antidepressants enhancing extracellular 5-HT levels, such as fluoxetine (Wong *et al*, 1974). Interestingly, a recent report has shown that treatment with fluoxetine, induces a robust increase in hippocampal pyramidal spine synapse density (Hajszan *et al*, 2005), indicating that this antidepressant may induce structural reorganization of neurons.

In order to better understand how neuronal structural plasticity in the mPFC is affected by depression and how this plasticity can be modulated by 5-HT pharmacological manipulation, we have investigated the structural relationship between 5-HT fibers and PSA-NCAM immunoreactive neurons, the presence of 5-HT receptors in PSA-NCAM expressing neurons of the mPFC and the effects of chronic fluoxetine treatment in the expression of PSA-NCAM in the mPFC.

MATERIALS AND METHODS

Animal Treatments and Histology

Twenty-six male Sprague-Dawley rats (4 months old, 320 ± 50 g Harlan Iberica) were used in this experiment. Animals were separated in the following groups: (i) six rats were used to study PSA-NCAM expression and its colocalization with 5-HT receptors using immunohistochemistry; (ii) 20 rats were used for fluoxetine and ondansetron treatment. All animal experimentation was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Rats 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). After perfusion, the brains were extracted and cryoprotected with 30% sucrose in PB. Coronal sections (50 μ m) were obtained with a sliding microtome and stored at -20°C in 30% glycerol; 30% ethylene glycol, 40% PB until used.

PSA-NCAM Immunohistochemistry

Tissue was processed 'free-floating' for immunohistochemistry as follows. Briefly, sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100°C . After cooling down the sections to room temperature, they were incubated with 10% methanol, 3% H_2O_2 in phosphate buffered saline (PBS) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 5% normal donkey serum (NDS) (Jackson Laboratories) in PBS with 0.2% Triton-X100

(Sigma) and were incubated overnight at room temperature in mouse monoclonal IgM Men-B anti-PSA-NCAM antibody (1:1400; generous gift of Dr G Rougon). After washing, sections were incubated for 30 min with donkey anti-mouse IgM biotinylated antibody (Jackson Laboratories, 1:250), followed by an avidin-biotin-peroxidase complex (ABC, Vector Laboratories) for 30 min in PBS. Color development was achieved by incubating with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) for 4 min. PBS containing 0.2% Triton-X100 and 3% NDS was used for primary and secondary antibodies dilution.

Pretreatment of the PSA-NCAM antibody with α -2,8-linked sialic polymer (colominic acid, Sigma) overnight, or the primary antibody omission during the immunohistochemistry prevented all the labeling in the mPFC.

All of the sections studied passed through all procedures simultaneously, to minimize any differences from immunohistochemical staining itself.

Double Immunofluorescence

In order to characterize the spatial relationship between 5-HT immunoreactive fibers and PSA-NCAM immunoreactive neurons, and the expression of 5-HT receptors (5-HT1A, 5-HT2A, and 5-HT3) in PSA-NCAM immunoreactive neurons and neuropil in the medial prefrontal cortex, we have performed double immunohistochemistry using an anti-PSA-NCAM antibody and antibodies against 5-HT and its receptors. In general, sections were processed as described above, but the endogenous peroxidase block was omitted. The sections were incubated overnight with mouse monoclonal IgM anti-PSA-NCAM antibody (Men-B, 1:1400) and one of the following primary IgG antibodies: Rabbit anti-5-HT (1:1000, Affinity), rabbit anti-5-HT1A (1:100, Santa Cruz Biotechnology H-119), mouse anti-5-HT2A (1:1000, Pharmigen), or rabbit anti-5-HT3 (1:250, Calbiochem). After washing, sections were incubated with donkey anti-mouse IgM, donkey anti-mouse IgG or donkey anti-rabbit IgG secondary antibodies conjugated with Alexa 488 or Alexa 555 (Molecular Probes, 1:200) in PBS containing 0.2% Triton X-100 and 3 % NDS. In the case of anti-5-HT2A the incubation with the two primary antibodies was made sequentially, first with the antibody against the receptor, washed and incubated with donkey anti-mouse IgG and then anti PSA-NCAM in order to avoid interactions between these two markers. The companies of origin have previously tested the specificity of the 5-HT and 5HT receptor antisera. Moreover, their specificity in rat tissue has been confirmed in different studies (Aznar *et al*, 2003; Morales *et al*, 1996; Morales and Bloom, 1997; Willins *et al*, 1997). Overnight incubation of 5-HT3 antibody with an excess of its immunogenic peptide resulted in a total absence of 5HT-3 immunostaining in the mPFC. Additional controls for the immunohistochemical procedure were carried out in our laboratory by omitting the primary or secondary antibodies in each step of the immunohistochemical protocol.

Observation and Quantification of Double-Labeled Cells

All sections processed for fluorescent immunohistochemistry were mounted on slides and coverslipped

using Permafluor mounting medium (Immunon/Shandon, Pittsburgh, PA). Then, the sections were observed under a confocal microscope (Leica TCS-SP2). Z-series of optical sections (1 μ m apart) were obtained using sequential scanning mode. These stacks were processed with LSM 5 Image Browser software. A 1-in-10 series of telencephalic sections from each animal were double-labeled as described. PSA-NCAM immunoreactive cells were first identified using conventional fluorescence microscopy. Then, a stack of confocal images covering all its three-dimensional extension was taken to confirm that PSA-NCAM labeling corresponded to cell somata. Fifty PSA-NCAM immunoreactive cells were analyzed in each case to determine the coexpression of PSA-NCAM and 5-HT receptors.

Pharmacological Treatments

Rats were divided into four groups ($n = 5$) and were treated (once daily) for 14 consecutive days, as follows. The first group was administered fluoxetine (10 mg/kg, intraperitoneal (i.p.), Sigma) and saline (30 min later). The second group received fluoxetine (10 mg/kg, i.p.) and the 5-HT₃ receptor antagonist ondansetron (2 mg/kg, i.p., generous gift of Glaxo-Smithkline), 30 min later. The third group received an injection of saline and then ondansetron (2 mg/kg), 30 min later. The fourth group received two injections of saline separated by 30 min. The volume injected in every i.p. injection was 500 μ l. All drugs used were dissolved in saline. After treatment, rats were killed and their brains were processed as described above.

Quantification of PSA-NCAM Immunoreactive Neurons and Neuropil Immunoreactivity

The number of PSA-NCAM expressing neurons in the different layers of the subdivisions of rat mPFC was estimated using a modified version of the fractionator method (West, 1993), as described before (Nacher *et al*, 2002a). We counted cells covering 100% of the sample area, that is, within each section, all labeled cells in the different subdivision and layers of the prefrontal cortex were counted. The fractionator sampling scheme refers to the methodology of examining one out of every 10 brain sections. Thus, our modification of the optical dissector combined with a 1:10 fractionator sampling is truly a modification of the optical fractionator method. 1:10 systematic-random series of sections covering the whole rostral to caudal extension of this structure were viewed on an Olympus CX41 microscope. Cell somata were identified and counted with a $\times 40$ objective. Cells appearing in the upper focal plane were omitted to prevent counting cell caps. The volume of the different areas analyzed was determined for each animal using the Cavalieri's principle (Gundersen and Jensen, 1987). Means were determined for each experimental group and the data were subjected to one-way ANOVAs followed by Student-Newman-Keuls *post hoc* tests.

In order to determine PSA-NCAM immunoreactivity intensity in the neuropil of medial prefrontal cortex, sections were examined under bright-field illumination, homogeneously lighted and digitalized using a CCD camera. Photographs were taken at $\times 20$ magnification. Gray levels were converted to optical densities (OD) using Image J

software (NIH). Means were determined for each experimental group and the data were subjected to one-way ANOVAs followed by Student-Newman-Keuls *post hoc* tests.

RESULTS

Body Weight

Body weight changes were monitored at the beginning (299.6 ± 3.4 g) and the end of the treatments (control: 356.0 ± 8.6 g; fluoxetine: 313.6 ± 4.9 g; fluoxetine + ondansetron: 325.0 ± 6.4 g; ondansetron: 357.2 ± 7.0 g). Fluoxetine treatment reduces significantly body weight gain and this reduction is prevented when ondansetron is coadministered with fluoxetine; ondansetron treatment alone did not interfere with body weight gain (One-way ANOVAs followed by Student-Newman-Keuls *post hoc* tests: $F_{4,20} = 694.154$, $p < 0.0001$).

Relationship between PSA-NCAM Immunoreactive Neurons and 5-HT Containing Fibers

The distribution of PSA-NCAM immunoreactivity in the mPFC coincides with that previously described. The immunoreactivity was located in neurons, mainly characterized as interneurons, and in the neuropil with a clear lamination in the staining (Varea *et al*, 2005). PSA-NCAM immunoreactive neurons were located in all the layers of the mPFC but they were most abundant in deep layers. In general, PSA-NCAM immunoreactivity in the mPFC neuropil was moderately intense in layer I, weaker in layers II and III and intense in layers V–VI.

We have analyzed whether 5-HT immunoreactive fibers were closely apposed to PSA-NCAM immunoreactive neurons in the rat mPFC using double immunostaining with these two markers. Fibers containing 5-HT tended to be more dense in layers I–III and they were sparse in the inner layers. Some PSA-NCAM immunoreactive neurons presented apposed 5-HT fibers (Figure 1a). However, most commonly, 5-HT immunoreactive fibers did not appear to contact PSA-NCAM immunoreactive neurons (Figure 1b).

5-HT Receptor Expression in PSA-NCAM Immunoreactive Neurons

We analyzed the presence of 5-HT_{1A}, 5-HT_{2A}, and 5-HT₃ receptors in PSA-NCAM immunoreactive neurons in the prefrontal cortex. These receptors were chosen because they are expressed by mPFC interneurons (Puig *et al*, 2004) and previous results from our laboratory have demonstrated that most PSA-NCAM immunoreactive cells in the rat mPFC are interneurons (Varea *et al*, 2005). 5-HT_{1A} and 5-HT_{2A} receptors are also expressed by pyramidal neurons, while 5-HT₃ receptor is only expressed by interneurons (Puig *et al*, 2004).

None of the PSA-NCAM immunoreactive neurons analyzed expressed 5-HT_{1A} (Figure 1c) or 5-HT_{2A} receptors (Figure 1d). 5-HT_{2A} was widely expressed by pyramidal neurons in this cerebral region (Figure 1e). By contrast, 94 % of the PSA-NCAM immunoreactive neurons also expressed the 5-HT₃ receptor (Figures 1f and g).

Figure 1 Confocal microscopic analysis of PSA-NCAM immunoreactive neurons in the mPFC. (a) PSA-NCAM immunoreactive neuron in the prelimbic cortex layer VI. Observe the presence of 5-HT fibers apposed to the neuron. (b) PSA-NCAM immunoreactive neuron in the cingulate cortex layer III. Note that 5-HT immunoreactive fibers (arrowhead) are not in the close vicinity of the neuronal soma. (c) Cingulate cortex layer VI. Double PSA-NCAM/5-HT1A immunohistochemistry. Observe the lack of colocalization between these two markers. Arrowhead indicates the presence of a PSA-NCAM immunoreactive neuronal soma. (d) Triple immunohistochemistry PSA-NCAM/5-HT2A/calbindin D28k in the cingulate cortex layer III. A PSA-NCAM immunoreactive neuron expresses calbindin but lacks 5-HT2A expression (arrowhead). Note the presence of two 5-HT2A immunoreactive cells on the bottom left side of the picture, which lack PSA-NCAM and calbindin D-28k immunoreactivity. (e) Calbindin D-28k/5-HT2A immunoreactive neuron in the cingulate cortex layer III. (f) PSA-NCAM/5-HT3 immunoreactive neuron in the prelimbic cortex layer V (arrowhead indicates the presence of two PSA-NCAM immunoreactive neurons displaying 5-HT3 immunoreactivity). (g) PSA-NCAM/5-HT3 immunoreactive neuron in the cingulate cortex layer III (arrowhead indicates the presence of 5-HT3 immunoreactive neurons displaying PSA-NCAM immunoreactivity). Scale bar: 10 μ m. All photographs in this figure correspond to single optical sections taken from Z-stacks.

Pharmacological Treatments

In order to check whether changes in extracellular levels of 5-HT could affect the expression of PSA-NCAM in the

mPFC, rats were chronically treated with fluoxetine for 14 days and PSA-NCAM immunoreactive neurons as well as neuropil staining were analyzed. As most PSA-NCAM immunoreactive neurons in the mPFC expressed 5-HT3 receptors we have also evaluated the effects of ondansetron, a 5-HT3 receptor antagonist, on PSA-NCAM expression, when used alone or in combination with fluoxetine.

Chronic fluoxetine treatment induced a general tendency towards an increase in the number of PSA-NCAM immunoreactive neurons in the mPFC (Figure 2a, $F_{3,21} = 5.36$, $p < 0.067$). Separated analysis of the different layers in the whole mPFC revealed that this increase in the number of immunoreactive neurons is only statistically significant in layers II–III. Coadministration of fluoxetine and ondansetron or ondansetron alone did not induce significant changes the number of PSA-NCAM expressing neurons in the whole mPFC; however, the numbers of PSA-NCAM immunoreactive neurons found in every layer after these treatments were significantly lower than that of fluoxetine-treated rats (Figure 2a, layer I: $F_{3,21} = 0.57$, $p < 0.6$; layers II–III: $F_{3,21} = 6.32$, $p < 0.002$; layers V–VI: $F_{3,21} = 4.34$, $p < 0.015$).

The individual analysis of the subdivisions of the mPFC revealed that the infralimbic cortex showed a statistically significant increase in the number of PSA-NCAM immunoreactive cells in whole infralimbic cortex and in layers II–III and a tendency towards an increase in the rest of the layers. Combined treatment with fluoxetine and ondansetron did not change the number of PSA-NCAM immunoreactive neurons in any of the layers analyzed. Similar results were obtained with ondansetron treatment alone, except in layers II–III, where a tendency towards a decrease in the number of labeled cells was observed. Animals treated with both ondansetron and fluoxetine (whole infralimbic cortex and layers II–III) or with ondansetron alone (whole infralimbic cortex and layers II–III and V–VI) exhibited significantly less PSA-NCAM immunoreactive neurons than those treated only with fluoxetine (Figure 2b, layer I: $F_{3,24} = 0.7$, $p < 0.55$; layers II–III: $F_{3,24} = 6.18$, $p < 0.0029$; layers V–VI: $F_{3,21} = 3.37$, $p < 0.024$).

In the prelimbic cortex (Figure 2c), no statistically significant differences in the number of PSA-NCAM expressing neurons were found between each of the treatments studied and the controls. However, the prelimbic cortex as a whole and layers II–III and V–VI showed a slight, but not statistically significant, increase in the number of PSA-NCAM immunoreactive neurons after chronic fluoxetine treatment. Animals treated with both ondansetron and fluoxetine exhibited significantly less PSA-NCAM immunoreactive neurons than those treated only with fluoxetine in layers V–VI (Figure 2c, layer I: $F_{3,24} = 0.75$, $p < 0.52$; layers II–III: $F_{3,24} = 1.64$, $p < 0.20$; layers V–VI: $F_{3,21} = 3.07$, $p < 0.047$).

Similar results were obtained in the cingulate cortex (Figure 2d). There was a slight, but not statistically significant, increase in the number of PSA-NCAM immunoreactive neurons after chronic fluoxetine treatment in the prelimbic cortex as a whole, and in layers II–III and V–VI. The rest of the treatments did not induce changes in the number of PSA-NCAM immunoreactive neurons in the cingulate cortex. However, fluoxetine-treated animals had significantly more PSA-NCAM immunoreactive neurons in

Figure 2 Graphs showing the changes in the number of PSA-NCAM immunoreactive neurons in medial prefrontal cortex: (a) Whole medial prefrontal cortex, (b) Infralimbic cortex, (c) Prelimbic cortex, (d) cingulate cortex. *Statistically significant, one-way ANOVA followed by Student–Newman–Keuls *post hoc* tests.

layers II–III than those treated with both fluoxetine and ondansetron. Animals treated with both ondansetron and fluoxetine exhibited significantly less PSA-NCAM immunoreactive neurons in the whole cingulate cortex and in layers II–III and V–VI than those treated only with fluoxetine (layer I: $F_{3,24} = 0.75$, $p < 0.52$; layer II–III: $F_{3,24} = 1.64$, $p < 0.20$; layer V–VI: $F_{3,21} = 3.07$, $p < 0.047$).

We also checked whether chronic treatment with fluoxetine affected the intensity of PSA-NCAM immunoreactivity in the neuropil. As a general feature, there was an increase in PSA-NCAM immunoreactivity in all the layers of all the subdivisions of the rat mPFC as a consequence of fluoxetine treatment, which was not observed when ondansetron was coadministered with fluoxetine (Figure 3).

In the infralimbic cortex the increase in PSA-NCAM immunoreactivity after fluoxetine treatment was statistically significant in every layer studied and it was prevented when ondansetron was coadministered with fluoxetine; ondansetron treatment alone did not change significantly the expression of PSA-NCAM in the infralimbic cortex, although there was a general tendency towards a decrease. Animals treated with fluoxetine–ondansetron or ondansetron alone showed significantly reduced immunoreactivity (in every layer studied) when compared with those treated only with fluoxetine (Figure 3a, layer I: $F_{3,19} = 6.31$, $p < 0.0037$; layer II: $F_{3,19} = 5.94$, $p < 0.0049$; layer III: $F_{3,19} = 8.57$, $p < 0.0008$; layer V: $F_{3,19} = 6.47$, $p < 0.0033$; layer VI: $F_{3,19} = 6.59$, $p < 0.0031$).

In the prelimbic cortex fluoxetine also induced a statistically significant increase in PSA-NCAM immunoreactivity in every layer studied, which was prevented when ondansetron was coadministered with fluoxetine; ondansetron treatment alone did not change significantly the expression of PSA-NCAM in any layer of the prelimbic cortex, although there was a general tendency towards a decrease. Animals treated with fluoxetine–ondansetron or ondansetron alone showed significantly reduced immunoreactivity (in every layer studied) when compared with those treated only with fluoxetine (Figure 3b, layer I: $F_{3,19} = 10.25$, $p < 0.0003$; layer II: $F_{3,19} = 9.25$, $p < 0.0006$; layer III: $F_{3,19} = 12.65$, $p < 0.0001$; layer V: $F_{3,19} = 12.09$, $p < 0.0001$; layer VI: $F_{3,19} = 9.84$, $p < 0.0004$).

Finally, in the neuropil of both the ventral and the dorsal cingulate cortices we have observed a statistically significant increase in PSA-NCAM expression in every layer after chronic fluoxetine treatment. These increases were not present when ondansetron was coadministered with fluoxetine. Interestingly, chronic ondansetron administration alone decreased significantly PSA-NCAM expression in layers II and III of the ventral cingulate cortex and there was also a tendency towards a decrease in the rest of the layers of both cingulate cortices. Animals treated with fluoxetine–ondansetron or ondansetron alone showed significantly reduced immunoreactivity (in every layer studied) when compared with those treated only with fluoxetine (ventral cingulate cortex: layer I: $F_{3,19} = 4.86$, $p < 0.011$; layer II:

Figure 3 Graphs representing the changes in the intensity of PSA-NCAM neuropil immunostaining in the mPFC. (a) Infralimbic cortex, (b) prelimbic cortex, (c) dorsal cingulate cortex. (d) Ventral cingulate cortex (asterisks in ondansetron bars indicate significant differences from control group). *Statistically significant, one-way ANOVA followed by Student–Newman–Keuls *post hoc* tests.

$F_{3,19} = 12.70$, $p < 0.0001$; layer III: $F_{3,19} = 16.53$, $p < 0.0001$; layer V: $F_{3,19} = 8.37$, $p < 0.0009$; layer VI: $F_{3,19} = 7.58$, $p < 0.0016$. dorsal cingulate cortex: layer I: $F_{3,19} = 4.14$, $p < 0.02$; layer II: $F_{3,19} = 4.25$, $p < 0.01$; layer III: $F_{3,19} = 7.44$, $p < 0.0017$; layer V: $F_{3,19} = 15.63$, $p < 0.0001$; layer VI: $F_{3,19} = 6.45$, $p < 0.003$).

DISCUSSION

This report describes a clear effect of chronic fluoxetine treatment on the expression of PSA-NCAM in the adult rat mPFC. We also provide evidence that PSA-NCAM immunoreactive neurons in this cortical region express 5-HT₃ receptors and that the effects of fluoxetine treatment on PSA-NCAM expression can be prevented by coadministration of a 5-HT₃ antagonist. The mechanisms by which 5-HT may modulate PSA-NCAM expression in the mPFC are discussed. We also analyze how these changes in PSA-NCAM expression may modulate neuronal structural plasticity. Finally, the putative functional consequences of this antidepressant action are discussed.

Involvement of 5-HT in the Modulation of PSA-NCAM Expression in the mPFC

Our results demonstrate that the expression of PSA-NCAM in the mPFC is modulated by chronic fluoxetine treatment.

As this compound acts inhibiting 5-HT reuptake and, consequently, increasing the extracellular concentrations of 5-HT (Wong *et al*, 1974), it is very likely that enhanced 5-HT binding to its receptors in certain mPFC neurons may induce the reported increase in PSA-NCAM expression. The adult rat mPFC is an important target of 5-HT action, because it receives a dense serotonergic innervation from the raphe nuclei (Azmitia and Segal, 1978; Steinbusch, 1981) and it contains several receptor subtypes for 5-HT (Pazos *et al*, 1985; Pazos and Palacios, 1985; Pompeiano *et al*, 1992). Moreover, the prefrontal cortex neurons project back to the brain nuclei that synthesize 5-HT, inducing its release (Jankowski and Sesack, 2004; Peyron *et al*, 1998; Takagishi and Chiba, 1991; Vertes, 2004). Previous reports have already demonstrated a link between PSA-NCAM expression and 5-HT: depletion of 5-HT reduces PSA-NCAM expression in the hippocampus, certain hypothalamic nuclei and the basal ganglia (Brezun and Daszuta, 1999; Brezun and Daszuta, 2000). Although the mechanism by which 5-HT modulates PSA-NCAM expression is not yet known, 5-HT may influence PSA-NCAM expression by acting directly on receptors located in certain neurons. Our results provide evidence that PSA-NCAM immunoreactive cells in the mPFC express 5-HT₃ receptors and thus it may be feasible that 5-HT could act directly through this type of receptor to upregulate PSA-NCAM expression. In fact, we have found that coadministration of fluoxetine and ondansetron (a 5-HT₃ receptor antagonist) prevents the

induction of PSA-NCAM expression induced by fluoxetine treatment. Moreover, chronic ondansetron administration alone is sufficient to induce a decrease in PSA-NCAM expression in certain layers of the cingulate cortex. Interestingly, recent results from our lab indicate that precisely these layers are the first to display changes in PSA-NCAM expression after chronic stress (Gomez-Climent and Nacher, unpublished results).

Fluoxetine appears to induce a general increase in PSA-NCAM expression, which is reflected by an increase in the number of positive neuronal somata and an increase in neuropil immunoreactivity. This may indicate an upregulation of PSA-NCAM expression in neuronal elements in which PSA-NCAM was not previously detected, because its level was under the detection limits of the technique employed, or to 'de novo' expression of PSA-NCAM in elements, which only expressed PSA-NCAM after fluoxetine treatment. We have not yet identified clearly the nature of PSA-NCAM expressing elements in the mPFC neuropil: they may correspond to neurites of intrinsic PSA-NCAM expressing neurons or they may correspond to processes coming from neurons located in areas that project to the mPFC.

We have found that 5-HT expressing fibers usually do not contact PSA-NCAM expressing neurons, suggesting that 5-HT action may not need direct contact between 5-HT expressing fibers and PSA-NCAM expressing cells. This result is in accordance with previous reports that indicate an extra-synaptic action of 5-HT, because this neurotransmitter is able to diffuse considerable distances from its point of release (De Miguel and Trueta, 2005) and consequently, once secreted, 5-HT can act on wide areas of the CNS. In fact, 5-HT receptors can be found widely distributed in the mPFC (Morales *et al*, 1996; Puig *et al*, 2004; Santana *et al*, 2004) and we detect increases in PSA-NCAM expression, specially in the neuropil, in every layer of the mPFC subdivisions. Thus, although the density of 5-HT fibers in superficial layers of the mPFC is higher, the increases in PSA-NCAM expression detected in deeper layers may also be explained by 5-HT action.

A previous report has described that PSA-NCAM expressing neurons in the rat mPFC are mainly interneurons and that pyramidal neurons do not appear to express this molecule (Varea *et al*, 2005). Interneurons in the mPFC can be targets of 5-HT action because they are known to express 5-HT_{1A}, 5-HT_{2A}, and 5-HT₃ receptors (Morales *et al*, 1996; Puig *et al*, 2004; Santana *et al*, 2004). Our present results indicate that 94% of PSA-NCAM expressing neurons in the mPFC express the 5-HT₃ receptor and that they lack 5-HT_{1A} and 5-HT_{2A} receptors, suggesting that 5-HT actions on PSA-NCAM expression occur through 5-HT₃ receptors. The 5-HT₃ receptor is a ligand-gated ionic channel (Derkach *et al*, 1989), permeable to monovalent cations (Peters and Lambert, 1989) and excitatory to interneurons (Puig *et al*, 2004). Previous reports have also identified 5-HT₃ expression in cortical interneurons expressing CCK, calbindin, and calretinin, although it is not present in parvalbumin expressing interneurons (Morales and Bloom, 1997). These results are in accordance with our previous report describing calbindin immunoreactivity in most mPFC PSA-NCAM expressing neurons and the absence of parvalbumin immunostaining in these cells (Varea *et al*, 2005).

The mechanism of 5-HT action on PSA-NCAM expression may be, at least partially, explained through the participation of 5-HT₃ receptors. The increase in the extracellular concentration of 5-HT induces depolarization of neurons through 5-HT₃ receptors, increasing neuronal activity (Puig *et al*, 2004), which might lead to increased PSA-NCAM expression.

Alternatively, the actions of 5-HT on PSA-NCAM expression may also be mediated by BDNF. Activation of 5-HT receptors can induce the expression of BDNF (see Mattson *et al* (2004) for review) and the interaction between this neurotrophin and PSA-NCAM is believed to be critical in regulating neuronal plasticity (Kiss *et al*, 2001).

Involvement of PSA-NCAM Expression in mPFC Neuronal Structural Plasticity

The presence of PSA-NCAM in the membrane of certain neurons may allow them to undergo plastic changes such as dendritic or spine remodeling. NCAM has the ability to incorporate long chains of polysialic acid (NCAM) conferring it anti-adhesive properties (Rutishauser, 1996). PSA-NCAM regulates CNS structural plasticity, allowing neurons to participate in plastic events such as axonal growth (Zhang *et al*, 1992) or synaptic reorganization (Miragall *et al*, 1988; Seki and Rutishauser, 1998). In adult animals, this molecule is expressed in cerebral regions that are undergoing some kind of structural plasticity, such as the hypothalamo-neurohypophyseal system (Theodosis *et al*, 1994), the olfactory bulb (Miragall *et al*, 1988) the piriform and entorhinal cortices (Seki and Arai, 1991a), the amygdala (Nacher *et al*, 2002b), and the hippocampus (Seki and Arai, 1991b). Previous reports have demonstrated the existence of structural plasticity in neurons of the mPFC, namely their ability to reorganize their structure under stress conditions (Brown *et al*, 2005; Cook and Wellman, 2004; Radley *et al*, 2004), or after corticosterone administration (Wellman, 2001). Moreover, the morphology of these neurons is also sensitive to alterations in blood pressure (Vega *et al*, 2004). PSA-NCAM is expressed in certain neurons and the neuropil of the rat mPFC (Varea *et al*, 2005), suggesting that some of the structural plasticity occurring in this cortical region may be mediated by this molecule.

Interestingly, although the expression of PSA-NCAM seems to be exclusive of mPFC interneurons (Varea *et al*, 2005), all the structural changes reported in the mPFC were only described in pyramidal neurons (Radley *et al*, 2005). However, at least some of these principal neurons are surrounded by PSA-NCAM immunoreactive puncta that may correspond to synapses (Varea *et al*, 2005). It is thus feasible that the presence of PSA-NCAM in certain synapses contacting mPFC pyramidal neurons may promote a transitory detachment, leading to a reorganization of pyramidal neuron dendrites. Alternatively, the increase in PSA-NCAM expression may indicate the presence of a sprouting phenomenon preceding or induced by a putative increase in the dendritic branching or in the number of dendritic spines in pyramidal neurons. In fact, chronic treatment with fluoxetine induces a robust increase in dendritic spine synapse density in CA1 and CA3 hippocampal pyramidal neurons (Hajszan *et al*, 2005) and similar effects may occur in the mPFC. Moreover, prefrontocortical

5-HT depletion, which may produce the opposite effects of fluoxetine, induces dendritic atrophy of mPFC pyramidal neurons (Perez-Vega *et al*, 2000). It is thus possible that 5-HT may act directly on pyramidal neurons, forcing them to modify its structure and that the increase in PSA-NCAM expression may be a response of interneurons to these structural changes.

Several studies have described opposite actions of chronic stress and antidepressant treatment on neuronal structural plasticity. One of the best studied regions is the hippocampus, in which chronic stress induces dendritic atrophy in pyramidal cells (McEwen, 2000). This atrophy is prevented by antidepressant treatment (Magarinos *et al*, 1999) and a recent report has described that fluoxetine treatment alone is capable of increasing spine synapses in these pyramidal neurons (Hajszan *et al*, 2005). As chronic stress also induces dendritic atrophy and spine loss in mPFC pyramidal neurons (Radley *et al*, 2004; Wellman, 2001), it is feasible that fluoxetine treatment may induce the opposite effect. The fact that chronic fluoxetine treatment induces an increase in synaptophysin (a protein specific of synapses) expression in the cerebral cortex (Rapp *et al*, 2004), gives support to this hypothesis. However, studies on the structure of mPFC neurons after antidepressant treatment should be performed to clarify this matter. Moreover, the identification of the phenotype and connectivity of the neurons that are expressing PSA-NCAM is a critical point that also deserves careful studies in the future. In any case, the observed changes in PSA-NCAM expression most likely reflect the reorganization of neuronal circuitry that is supposed to occur after antidepressive treatment (Castren, 2005).

Functional Implications of Fluoxetine Treatment and Structural Plasticity

Although the suggestion that mood disorders were caused by a neurochemical imbalance has been widely accepted over the last few decades, recent evidences have lead to the emergence of a new hypothesis, which suggests that dysfunction in neuronal plasticity may underlie the etiology of depression (Castren, 2005; Mattson *et al*, 2004). The basis for the neurochemical hypothesis of depression came from the finding that certain antidepressants act increasing the extracellular concentrations of 5-HT and noradrenaline in the CNS (monoamine hypothesis). However, the relationship between depression and monoamine concentrations appeared to be more complex than previously thought. Although the effects of antidepressants on monoamine metabolism occur rapidly, the therapeutic effects of antidepressants usually need weeks to take place (Nestler, 1998). This delay and the recent finding that antidepressants induce neuronal structural plasticity add support to the neuroplastic hypothesis.

Although we are still far from understanding the cellular bases of depression, the presence of PSA-NCAM and its modulation by fluoxetine open a new perspective in the study of this mood disorder. Of particular interest is the recent development of PSA-NCAM mimotope peptides, which may be promising therapeutic drugs for the treatment of mood disorders (Cambon *et al*, 2004; Torregrossa *et al*, 2004). Future directions should include

the study of PSA-NCAM expression in the brains of depressed patients and research directed to deepen into the molecular cascades leading to the enhanced PSA-NCAM expression found after antidepressant treatment.

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