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International Journal of Neuropsychopharmacology, Page 1 of 12. © CINP 2014
doi:10.1017/S1461145714000406

ARTICLE

Chronic fluoxetine treatment alters the structure, connectivity and plasticity of cortical interneurons

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Abstract

Novel hypotheses suggest that antidepressants, such as the selective serotonin reuptake inhibitor fluoxetine, induce neuronal structural plasticity, resembling that of the juvenile brain, although the underlying mechanisms of this reopening of the critical periods still remain unclear. However, recent studies suggest that inhibitory networks play an important role in this structural plasticity induced by fluoxetine. For this reason we have analysed the effects of a chronic fluoxetine treatment in the hippocampus and medial prefrontal cortex (mPFC) of transgenic mice displaying eGFP labelled interneurons. We have found an increase in the expression of molecules related to critical period plasticity, such as the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), GAD67/65 and synaptophysin, as well as a reduction in the number of parvalbumin expressing interneurons surrounded by perineuronal nets. We have also described a trend towards decrease in the perisomatic inhibitory puncta on pyramidal neurons in the mPFC and an increase in the density of inhibitory puncta on eGFP interneurons. Finally, we have found that chronic fluoxetine treatment affects the structure of interneurons in the mPFC, increasing their dendritic spine density. The present study provides evidence indicating that fluoxetine promotes structural changes in the inhibitory neurons of the adult cerebral cortex, probably through alterations in plasticity-related molecules of neurons or the extracellular matrix surrounding them, which are present in interneurons and are known to be crucial for the development of the critical periods of plasticity in the juvenile brain.

Received 11 November 2013; Reviewed 13 December 2013; Revised 16 February 2014; Accepted 6 March 2014

Key words: Critical period plasticity, fluoxetine, interneurons, perineuronal nets, PSA-NCAM.

Introduction

Neuronal plasticity is required for the adaptation of the brain to face a changing external environment. Several lines of evidence during the last decade have suggested that antidepressants may act by promoting this plasticity (Duman et al., 2000; Castrén, 2005). The antidepressant fluoxetine, a selective serotonin reuptake inhibitor, promotes different kinds of plasticity in the adult CNS, including increased hippocampal neurogenesis (Malberg et al., 2000), LTP induction (Rubio et al., 2013) or remodelling in the structure of pyramidal neurons (Hajszan et al., 2005; Guirado et al., 2009). However, to date most of the research on the effects of antidepressants has been focused on excitatory neural networks, leaving their action on inhibitory neurons almost unexplored (Chen et al., 2011).

Recent research has promoted the development of a new perspective on our understanding of the mode of action of antidepressants: The structural plasticity induced by antidepressants and particularly fluoxetine, resembles that naturally occurring during the critical periods of development. Therefore, fluoxetine may act by promoting a 'dematuration' in certain regions of the adult brain, such as the limbic system (Kobayashi et al., 2010). Moreover, research on the visual cortex has, in fact, demonstrated that the plasticity induced by fluoxetine is similar to that observed during critical periods, when the neuronal wiring of this cortical region is finally established (Maya-Vetencourt et al., 2008).

Cell adhesion molecules and several components of the extracellular matrix have been shown to be critical for structural plasticity in the CNS (Sandi, 2004; Dityatev et al., 2010; Tiraboschi et al., 2013). In this regard, the polysialylated form of the neural cell

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adhesion molecule (PSA-NCAM) and the perineuronal nets (PNNs) are interesting candidates to mediate these changes. These molecules have been both demonstrated to play a key role in synaptogenesis and neurite remodelling (Rutishauser, 2008; Howell and Gottschall, 2012). While PSA-NCAM is widely considered a marker of developing neurons, which expression decreases as development progresses (Probstmeier et al., 1994; Kurosawa et al., 1997; Oltmann-Norden et al., 2008), PNNs are considered indicators of neuronal maturation and, consequently, the number of neurons expressing these specialized regions of the extracellular matrix increases with age (McRae et al., 2007; Nowicka et al., 2009).

Fluoxetine treatment induces a similar type of juvenile plasticity in the adult amygdala, allowing the erasure of fear memories. This reopening of the critical period was correlated with an increase in the expression of PSA-NCAM and with a reduction of the ratio of PNNs expressing parvalbumin (Karpova et al., 2011). In agreement with this plasticity-promoting role, the enzymatic removal of these molecules has an impact on the critical period plasticity. Removal of PSA-NCAM accelerates the maturation of the visual cortex and the closure of the critical period (Di Cristo et al., 2007), while removal of PNNs delays this maturation and maintains open the critical period for a longer time (Pizzorusso et al., 2002). However, little is known of the effect of this antidepressant in the hippocampus or the mPFC, two regions critically involved in the aetiopathology of major depression.

It is interesting to note that both PSA-NCAM and PNNs are found in subsets of interneurons in the adult cerebral cortex. PNNs mainly appear surrounding parvalbumin expressing interneurons (Brückner et al., 1994), and PSA-NCAM is expressed mainly by calbindin expressing interneurons, although it is also found in a lower proportion in the somata of other interneuronal populations and in parvalbumin expressing perisomatic puncta surrounding pyramidal neurons (Gómez-Climent et al., 2010; Castillo-Gómez et al., 2011). This suggests that interneurons may play a key role in the structural plasticity induced by the antidepressant fluoxetine. In fact, a recent report has demonstrated that chronic treatment with fluoxetine alters GABA release from synapses formed by fast-spiking cells, resulting in the disruption of γ oscillations (Méndez et al., 2012).

Thus, in order to better understand how fluoxetine influences the structural plasticity and the connectivity of interneurons in the mPFC and the hippocampus, we have analysed the effects of fluoxetine on: (i) the ratio of PNNs surrounding PV interneurons, (ii) the expression of PSA-NCAM and synaptic molecules related to inhibitory neurotransmission in the neuropil, (iii) the perisomatic innervation of pyramidal and inhibitory neurons, and (iv) the structure of interneurons in these two cortical regions.

Method

Animal treatments

Twelve male GIN (GFP-expressing inhibitory neurons, Tg(GadGFP)45704Swn) (Oliva et al., 2000), three-month-old mice were used in this study. They were maintained in standard conditions of light (12 h cycles) and temperature, with no limit in the access to food or water. All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes 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. Mice received daily i.p. injections either with the antidepressant fluoxetine ($n=6$, 20 mg/kg), or with saline solution ($n=6$), over 14 days (once daily at 10.00 am). Previous studies have shown that this dose and length of treatment produces increases in the expression of plasticity-related molecules in the regions analysed (Umathe et al., 2011; Yu et al., 2011; Méndez et al., 2012). The day after these treatments, mice were perfused transcardially under deep chloral hydrate anaesthesia (4%, 0.01 ml/g), first with saline and then with 4% paraformaldehyde in sodium phosphate buffer (PB 0.1 M, pH 7.4). After perfusion, the brains were extracted and stored in PB 0.1 M and sodium azide 0.05%. The two hemispheres were separated, then one hemisphere was cut into 50 μm thick sections, while the other was cut into 100 μm thick sections, both with a vibratome (Leica VT 1000E, Leica). The sections were collected in six and three subseries, respectively, and stored at 4 °C in PB 0.1 M and sodium azide 0.05% until used.

Immunohistochemistry

Tissue was processed free-floating for fluorescence immunohistochemistry. Sections were washed in phosphate buffered saline (PBS), then slices were incubated in 10% normal donkey serum (NDS) (Abcys SA), 0.2% Triton-X100 (Sigma) in PBS for 1 h. Sections were then incubated for 48 h at 4 °C with different primary antibody cocktails diluted in PBS – 0.2% Triton-X100 (see Table 1). After washing, sections were incubated for 2 h at room temperature with different secondary antibody cocktails also diluted in PBS – 0.2% Triton-X100 (see Table 1). Finally, sections were washed in PB 0.1 M, mounted on slides and coverslipped using fluorescence mounting medium (Dako).

In the present study we used only commercial antibodies whose specificity was proved by the provider. Additionally, when the antigen used for generating the primary antibody was available, we first co-incubated them in accordance with the immunohistochemical protocol in order to block the binding of these antibodies to the tissue antigens. In the cases of anti-GAD6 and anti-CAMKII antibodies no access was available to the

Table 1. List of primary and secondary antibodies used in the study

	Dilution	Company
Primary antibodies		
Monoclonal mouse anti-PSA-NCAM	1:2000	Millipore
Polyclonal guinea pig antivesicular glutamate transporter 1	1:2000	Millipore
Polyclonal rabbit anti-synaptophysin	1:1000	Millipore
Monoclonal mouse anti-glutamic acid decarboxylase 65/67	1:500	DSHB
Polyclonal guinea pig anti-parvalbumin	1:2000	Synaptic Systems
Polyclonal chicken anti-GFP	1:1000	Millipore
WFA lectin biotin-conjugated	1:200	Sigma
Monocolonal mouse anti-CaMKII	1:500	Abcam
Secondary antibodies		
Goat anti-mouse A555-conjugated	1:400	Invitrogen
Donkey anti-rabbit A555-conjugated	1:400	Invitrogen
Goat anti-guinea pig A647-conjugated	1:400	Invitrogen
Donkey anti-chicken A488-conjugated	1:400	Invitrogen
Streptavidin A647-conjugated	1:400	Invitrogen
Donkey anti-mouse DL649-conjugated	1:400	Jackson
Donkey anti-mouse A488-conjugated	1:400	Invitrogen

antigens used for their generation. However, these antibodies are included in the JCN AntibodyDatabase: http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%2910969861/homepage/jcn_antibody_database.htm. The antibodies on this list have been described and characterized adequately according to the strict guidelines of the journal, including appropriate controls. The primary antibodies against VGLUT1 and PV were pre-adsorbed with the peptides used for their generation by their commercial source: AG208 (Millipore) and SySy 195-0P (synaptic systems), respectively. The primary antibody against SYN was pre-adsorbed with a synaptophysin recombinant protein (H00006855-P01, Novus). The antibody against PSA-NCAM was co-incubated with alpha-2,8-sialic acid (Colominic acid, Sigma). The performance of the immunohistochemical procedure using these blocked antibodies resulted in a total absence of immunostaining. The *Wisteria floribunda* lectin was pre-adbsorbed with N-Acetyl-D-galactosamine (Sigma, A2795). The performance of the histochemical procedure using this blocked lectin also resulted in a total absence of staining.

Analysis of immunoreactive puncta in single confocal planes

We analysed the density of puncta expressing different markers in single confocal planes (Olympus FV10i) of different regions of the mouse brain, as previously described (Guirado et al., 2012). We imaged an area located within layer V of the medial prefrontal cortex (mPFC). In this layer reside the pyramidal neurons that provide the main output projection of this region, which project extensively to the striatum (Lévesque and Parent, 1998). We analysed the different regions of the mPFC in two different sections: the prelimbic (PrL) and the infralimbic cortices (IL; in sections corresponding to Bregma +1.78 mm) and the ventral and dorsal cingulate cortices (Cg1 and Cg2 respectively; Bregma +1.1 mm). Different regions and strata of the hippocampus were also analysed (Bregma −2.18 mm): the hilus and the molecular layer of the dentate gyrus, the stratum lacunosum-moleculare, radiatum and oriens of CA1 and the stratum lucidum of CA3. Confocal *z*-stacks of the superficial layers of the slices, for optimal penetration levels for antibody recognition were selected.

On these planes, three small areas of the neuropil (505 μm^2) were selected for analysis, in order to avoid blood vessels and cell somata. Images were processed using ImageJ software. The background was subtracted with a rolling value of 50, converted to 8-bit deep images and binarized using a determined threshold value. This value depended on the marker and the area analysed and was kept the same for all images with the same marker and area. The images were then processed with a blur filter to reduce noise and separate closely apposed puncta. Finally, the number of the resulting dots per region was counted (Fig. S1).

Analysis of the density of perisomatic puncta

We analysed the density of puncta surrounding the cell somata of pyramidal neurons and interneurons using a similar methodology to that described above. In the mPFC, between 20 and 30 pyramidal neurons were imaged per animal in three different sections and similar numbers where analysed in the CA1 region of the hippocampus. However, only between 10 and 15 interneurons were counted per region (also both in the mPFC and the hippocampus), since their density was more limited. Images were processed using ImageJ. The profile of the plasmatic membrane of every soma was delimited manually, and then the selection was enlarged 1 μm in order to cover the area surrounding the somata. The selected area was processed for binarization as described above.

Analysis of spine density

To study the spine density of interneurons we selected individual dendrites from eGFP-expressing neurons in deep layers of the mPFC and in the CA1 region of the hippocampus. Stacks of confocal images were obtained with a 63× objective and an additional 3.5 digital zoom. The spines were counted in three dendritic fragments (around 60 μm each) expanding until 180 μm from the soma. The length of every dendritic fragment was measured and the data was expressed in density of spines per 10 μm.

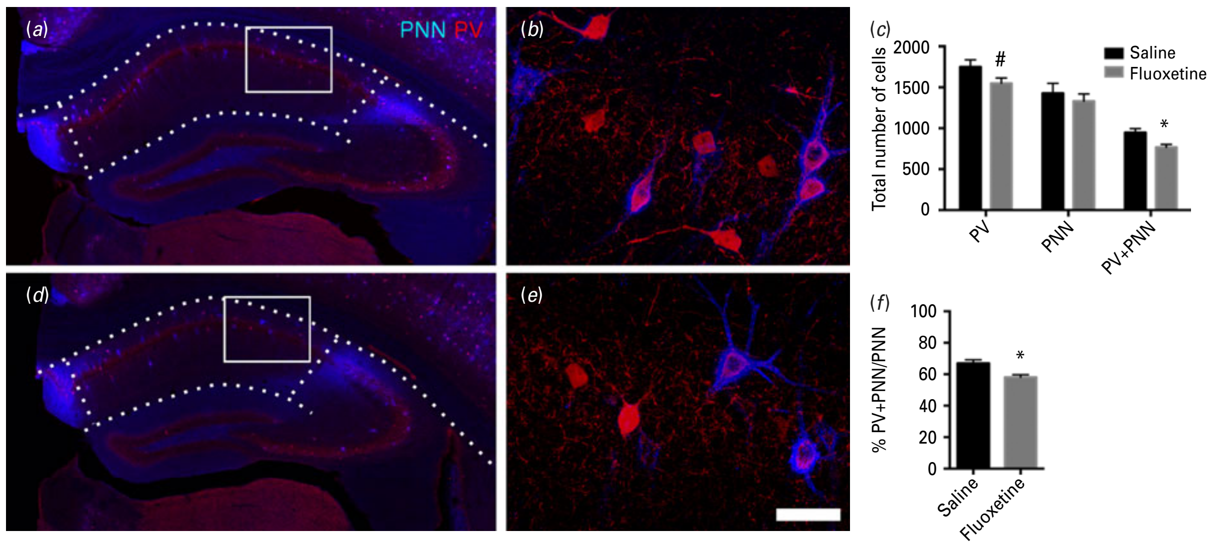

Fig. 1. Expression of PV and PNNs, after chronic fluoxetine treatment, in the hippocampus. (*a* & *d*): Single confocal planes showing the expression of PV and the presence of PNNs in the CA1 region of the hippocampus of mice treated either with saline (*a*) or fluoxetine (*d*). (*b* & *e*): Higher magnification microphotographs showing differences in the number of neurons co-expressing both markers. (*c* & *f*): Graphs showing the changes in cells expressing each of the markers, expressing both markers and the ratio of total PNNs surrounding PV-expressing neurons (Unpaired *T*-test, *p*-values: #<0.1, *<0.05). Scale bar 377 μm in A and D, 41 μm in (*b*) and (*e*). PNNs: perineuronal nets; PV: parvalbumin.

Estimation of number of cells

We also estimated the number of cells expressing parvalbumin (PV) or surrounded by perineuronal nets (PNN) as previously described (Nacher et al., 2002b). In brief, we analysed sections selected by a 1:6 fractionator sampling covering the whole rostral to caudal extension of the mPFC and the hippocampus CA1.

Statistics

We have used two-way ANOVAs to analyse the data whenever we had to consider more than one factor, as in the analysis of the density of immunoreactive puncta in different subregions or the density of spines at different distances from the soma. For comparing two means with only one factor, we used unpaired *t*-tests, as in the study of the perisomatic innervation and the total number of cells expressing PV, surrounded by PNNs, PV/PNNs positive cells, or the ratio of PNNs surrounding PV immunoreactive neurons. For all statistics the number of animals in each group was considered as the '*n*'.

Results

Chronic fluoxetine treatment alters the expression of plasticity-related molecules in interneurons of the mPFC and the hippocampus

We found that animals treated with fluoxetine displayed a non-significant trend towards a decreased number of PV-expressing interneurons in the CA1 region of the hippocampus ($p=0.098$; Fig. 1). In addition, the number of PV interneurons expressing PNNs was significantly reduced ($p=0.014$). Moreover, another parameter typically measured to evaluate the maturation degree of a cerebral region (Karpova et al., 2011) was also affected—the ratio of neurons surrounded by PNNs that co-expressed PV—was significantly reduced ($p=0.016$). In the mPFC of mice treated with fluoxetine we found a non-significant trend towards a decrease in the number of neurons surrounded by PNNs ($p=0.094$; Fig. 2) and a significant reduction in the number of PV interneurons expressing PNN ($p=0.042$). However, the ratio mentioned above was not affected in this region.

We found that after chronic fluoxetine treatment there are significant increases in the density of PSA-NCAM-expressing puncta in the strata lacunosum-moleculare ($p=0.040$; Fig. 3), radiatum ($p=0.048$) and oriens ($p=0.002$). Similar increases were found in the density of synaptophysin (SYN)-expressing puncta in the strata lucidum ($p=0.045$), moleculare ($p=0.008$), oriens ($p=0.027$) and radiatum ($p=0.016$). We also found a significant increase ($p=0.016$) in the density of glutamic acid decarboxylase 67/65 (GAD6)-expressing puncta in the hilus. However, we did not find any change in the density of the vesicular glutamate transporter 1 (VGluT1)-expressing puncta in any of the areas analysed. In the mPFC we only found a non-significant trend towards an increase in the density of neuropil-expressing PSA-NCAM ($p=0.060$; Fig. 3) and SYN ($p=0.073$) in the Cg2 region, in animals chronically treated with fluoxetine. Furthermore, we did not find any changes in the expression of GAD6 or of VGluT1 in any region of the mPFC.

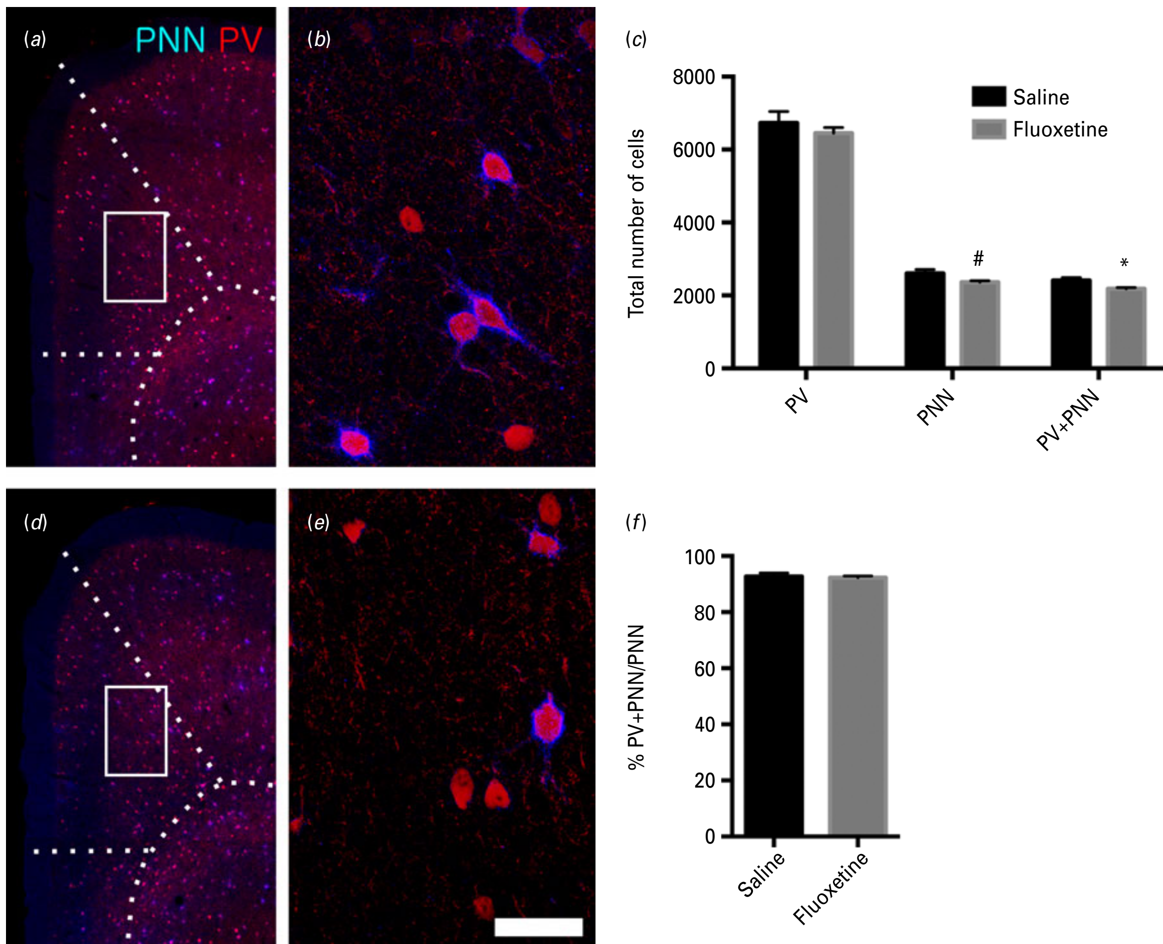

Fig. 2. Expression of PV and PNN after chronic fluoxetine treatment in the mPFC. (*a* & *d*): Single confocal planes showing the expression of PV and the presence of PNNs in the mPFC of mice treated either with saline (*a*) or fluoxetine (*d*). (*b* & *e*): Higher magnification microphotographs showing differences in the number of neurons co-expressing both markers. (*c* & *f*): Graphs showing the changes in cells expressing each of the markers, both markers and the ratio of total PNNs surrounding PV-expressing neurons (Unpaired *T*-test, *p*-values: #<0.1, *<0.05). Scale bar 286 μm in (*a*) and (*d*), 41 μm in (*b*) and (*e*). PNNs: perineuronal nets; PV: parvalbumin.

Chronic fluoxetine treatment increases the number of inhibitory perisomatic puncta on interneurons but not on pyramidal neurons

We analysed the density of PV positive puncta surrounding the somata of pyramidal neurons and its co-localization with SYN (Fig. 4). In the hippocampus we did not observe any change in the density of PV- or SYN- expressing puncta or in the density of PV puncta expressing SYN after fluoxetine treatment. However, in the mPFC we found a non-significant trend towards a decrease in the density of perisomatic-PV-puncta-expressing SYN (p=0.080; Fig. 4).

We observed an increase in the density of GAD6-expressing puncta surrounding the somata of interneurons expressing eGFP in the CA1 region of the hippocampus of animals treated with fluoxetine (p=0.015; Fig. 4). Although there were no changes in the density of puncta expressing SYN surrounding the somata of these interneurons, we also found an increase in the density of perisomatic-GAD6-puncta-expressing SYN (p=0.028; Fig. 4). However, in the mPFC we found no changes in the density of perisomatic puncta on interneurons.

Chronic fluoxetine treatment increases the dendritic spine density in interneurons of the mPFC

We described recently that this subpopulation of eGFP interneurons display spines on their dendrites (Gómez-Climent et al., 2010), whose dynamics are influenced by PSA-NCAM expression (Guirado et al., 2013). As some of these interneurons co-express PSA-NCAM, we studied both subpopulations: eGFP interneurons expressing PSA-NCAM and those lacking this molecule. We compared the dendritic spine density of interneurons expressing PSA-NCAM in animals treated with fluoxetine and those injected with saline and we found that, in our paradigm, fluoxetine does not affect the dendritic spine density of these interneurons in the

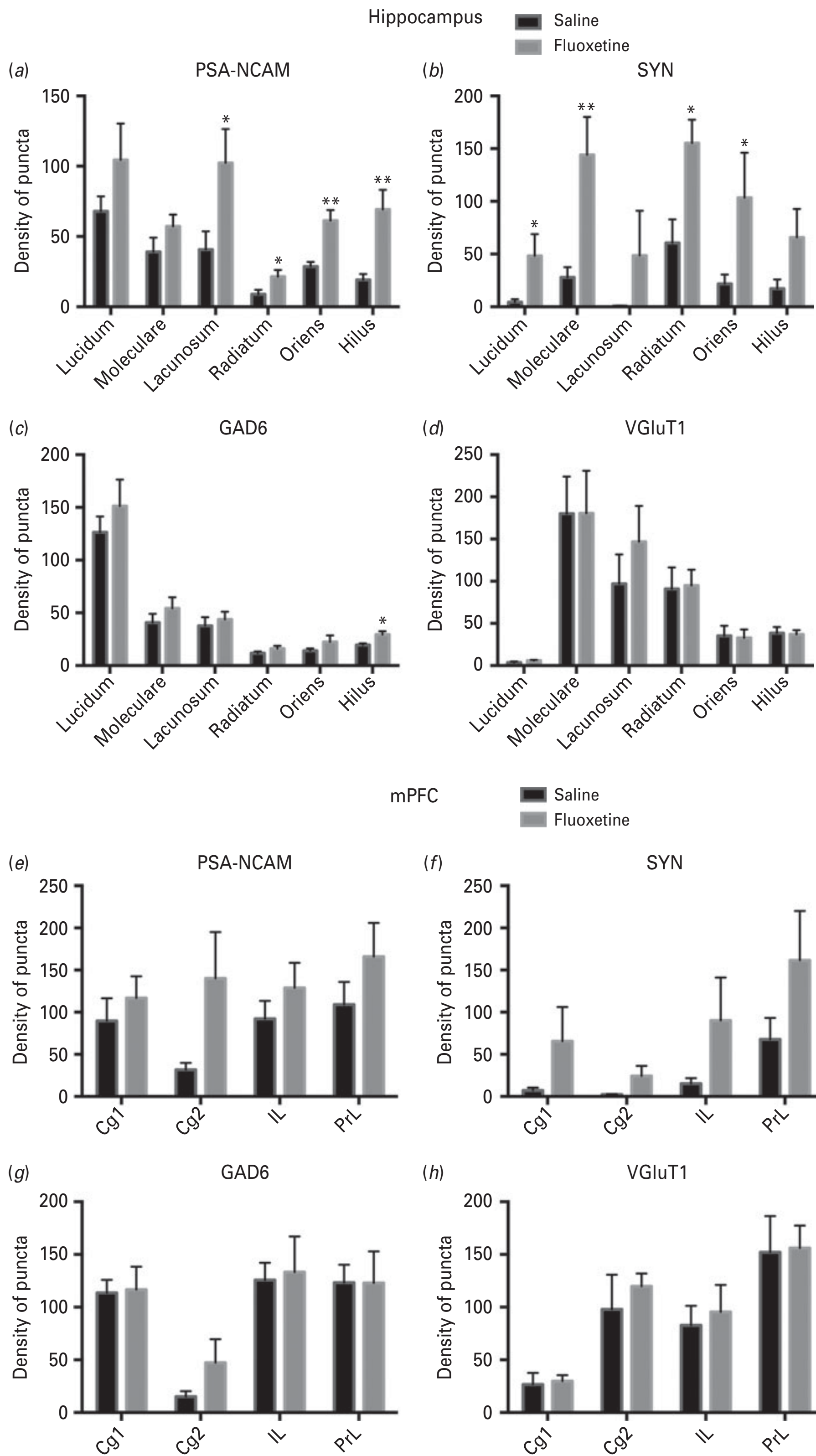

Fig. 3. Expression of molecules related to neuronal plasticity in the different strata of the hippocampus (*a*–*d*) and the mPFC (*e*–*h*). (*a*): Graphs showing the density of puncta-expressing PSA-NCAM. (*b*): Graphs showing the density of puncta-expressing SYN. (*c*): Graphs showing the density of puncta-expressing GAD6. (*d*): Graphs showing the density of puncta-expressing VGluT1 (One-way ANOVA, *p*-values: *<0.05, **<0.01). (*e*): Graphs showing the density of puncta-expressing PSA-NCAM. (*f*): Graphs showing the density of puncta-expressing SYN. (*g*): Graphs showing the density of puncta-expressing GAD6. (*h*): Graphs showing the density of puncta-expressing VGluT1. (One-way ANOVA, *p*-values: #<0.1). GAD6: Glutamic acid decarboxylase 65/67; SYN: synaptophysin; VGluT1: vesicular glutamate transporter 1.

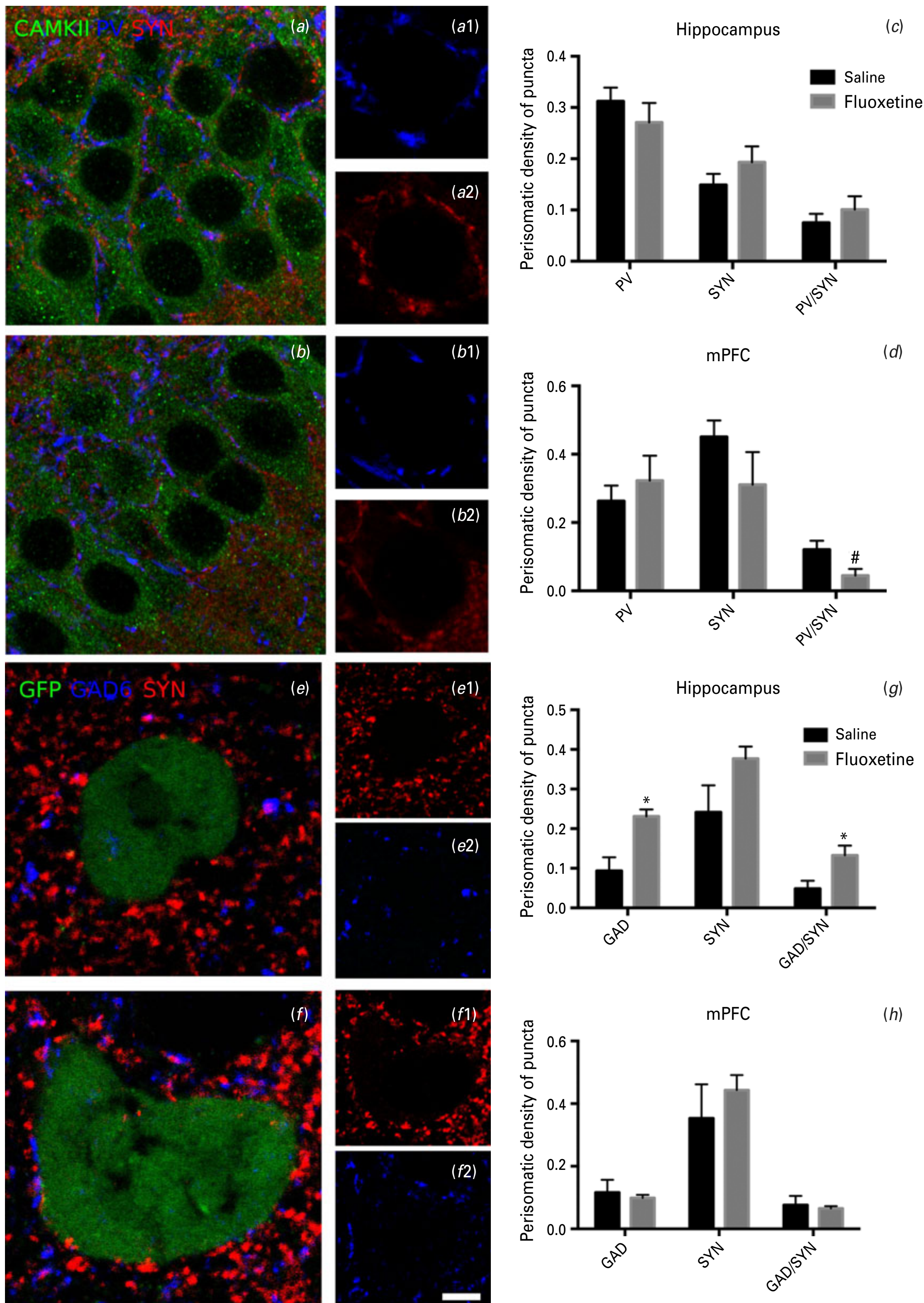

Fig. 4. (*a* & *b*): Effects of chronic fluoxetine treatment on the perisomatic innervation of pyramidal neurons. Single confocal planes of the CA1 stratum pyramidale of the hippocampus showing CAMKIIα-expressing pyramidal neuron somata (green) and perisomatic-puncta-expressing PV (blue) or SYN (red) in saline (*a*) and fluoxetine treated (*b*) mice. (*a1–a2* & *b1–b2*): Single confocal planes showing PV- (*a1, b1*), and SYN- (*a2, b2*) expressing puncta surrounding the somata of a pyramidal neuron of saline (*a* series) and fluoxetine treated (*b* series) mice. (*c* & *d*): Histograms showing the perisomatic density of the puncta-expressing the different markers surrounding pyramidal neurons in the hippocampus and the mPFC. (Unpaired *T*-test, *p*-values: #<0.1). (*e* & *f*): Effects of chronic fluoxetine treatment on the perisomatic innervation of interneurons. Single confocal planes of eGFP-expressing interneurons

hippocampus. Similarly, we did not find changes when comparing eGFP-expressing interneurons lacking PSA-NCAM, after fluoxetine treatment. However, we replicated our previous results, confirming that interneurons expressing PSA-NCAM have a reduced dendritic spine density compared with interneurons lacking this molecule (Fig. S2).

However, in the mPFC, where eGFP-expressing interneurons always lack PSA-NCAM expression, we found that animals treated with fluoxetine had a significant increase in dendritic spine density in these interneurons ($p=0.005$). When analysing the different segments, we found that these changes were especially more intense in the distal segment of the dendrites ($p=0.008$; Fig. 5).

Discussion

In the current study, we described the effects of chronic treatment with fluoxetine on different parameters affecting inhibitory networks in the hippocampus and the mPFC. This antidepressant increases the number of inhibitory perisomatic puncta on interneurons, but not on pyramidal neurons, and increases the density of dendritic spines in interneurons of the mPFC. Fluoxetine induces these changes in inhibitory networks and through putative mechanisms alters the expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) and the number of perineuronal nets (PNNs). Both molecules (PSA-NCAM and PNNs) play an important role in the plasticity associated with the development and closure of critical period plasticity, are mainly associated with interneurons in the adult cerebral cortex and are considered markers of neuronal development and of neuronal maturation, respectively.

We have found that the chronic fluoxetine treatment used in our study does not induce significant alterations in the density of perisomatic puncta on the mPFC pyramidal neurons, although there was a trend for a decrease in the number of puncta co-expressing PV and SYN. These results are in contrast to those of a recent report, which has found that chronic treatment with fluoxetine alters GABA release from synapses formed by hippocampal fast-spiking cells, resulting in the disruption of γ oscillations (Méndez et al., 2012). It is possible that the lack of significant differences in the density of perisomatic puncta found in our study may be due to the fact that, despite the fact that the doses used were the same, our mice were treated with fluoxetine for two weeks only while the rats in Méndez et al. (2012) study received the treatment for three weeks.

We found that, similar to pyramidal neurons, the somata of interneurons expressing eGFP are also surrounded by puncta expressing the inhibitory marker GAD6 and that chronic fluoxetine treatment induces a dramatic increase in the density of these perisomatic puncta in the hippocampus, but not in the mPFC. Although the co-localization with synaptophysin indicates that these perisomatic inhibitory puncta may correspond to active synapses, it is still complicated to discuss the functional implications of their increase via fluoxetine. It would be necessary to perform new experiments including electron microscopy to clarify which subpopulation/s are affected by this putative increase in perisomatic inhibition.

Our study shows for the first time that chronic treatment with fluoxetine alters the structure of interneurons. Previous studies have demonstrated that fluoxetine treatment increases the dendritic spine density in pyramidal neurons of the hippocampus (Hajszan et al., 2005) and the somatosensory cortex (Guirado et al., 2009), but there is no information on its effect on the mPFC. We found that fluoxetine induces an increase in the density of dendritic spines in eGFP-labelled interneurons in the mPFC, but not in those of the hippocampal CA1 region. A recent report by our laboratory has revealed that these spines receive mainly excitatory synapses (Guirado et al., 2013). In the mPFC of the strain of mice used in the present study, eGFP-labelled interneurons correspond to Martinotti cells (Gilabert-Juan et al., 2013), which innervate the distal portion of the dendritic arbour of pyramidal neurons (Markram et al., 2004). If the new synapses that these interneurons receive on their spines are functional, then they may enhance the excitatory input that these interneurons receive and, in turn, enhance the inhibition produced on mPFC pyramidal neurons. This would be in accordance with previous reports describing increased expression of molecules involved in inhibitory neurotransmission in rats chronically treated with fluoxetine (Varea et al., 2007a; Guirado et al., 2012; Tiraboschi et al., 2013). Since a previous report has described decreased cortical inhibition in the visual cortex of rats chronically treated with fluoxetine (Maya-Vetencourt et al., 2008), it is possible that the changes in the structure of interneurons and the

located in the CA1 region of the hippocampus showing eGFP in their somata (green) and perisomatic-puncta-expressing GAD6 (blue) or SYN (red) in saline (*e*) and fluoxetine treated (*f*) mice. (*e1–e2* & *f1–f2*): Single confocal planes showing SYN- (*e1, f1*) and GAD6- (*e2, f2*) expressing puncta surrounding the somata of an eGFP-expressing interneuron of saline (*e* series) and fluoxetine treated (*f* series) mice. (*g* & *h*): Histograms showing the perisomatic density of the puncta-expressing the different markers surrounding eGFP-expressing interneurons in the hippocampus and the mPFC. (Unpaired *T*-test, *p*-values: *<0.05). Scale bar 5 μm in (*a*) and (*b*), 2.5 μm in (*a1–a2* and *b1–b2*), 20 μm in (*e*) and (*f*), 6.4 μm in (*e1–e2* and *f1–f2*). CAMKIIα: anti-a subunit of the Ca2 +/calmodulin dependent protein kinase II; GAD6: glutamic acid decarboxylase 65/67; PV: parvalbumin; SYN: synaptophysin.

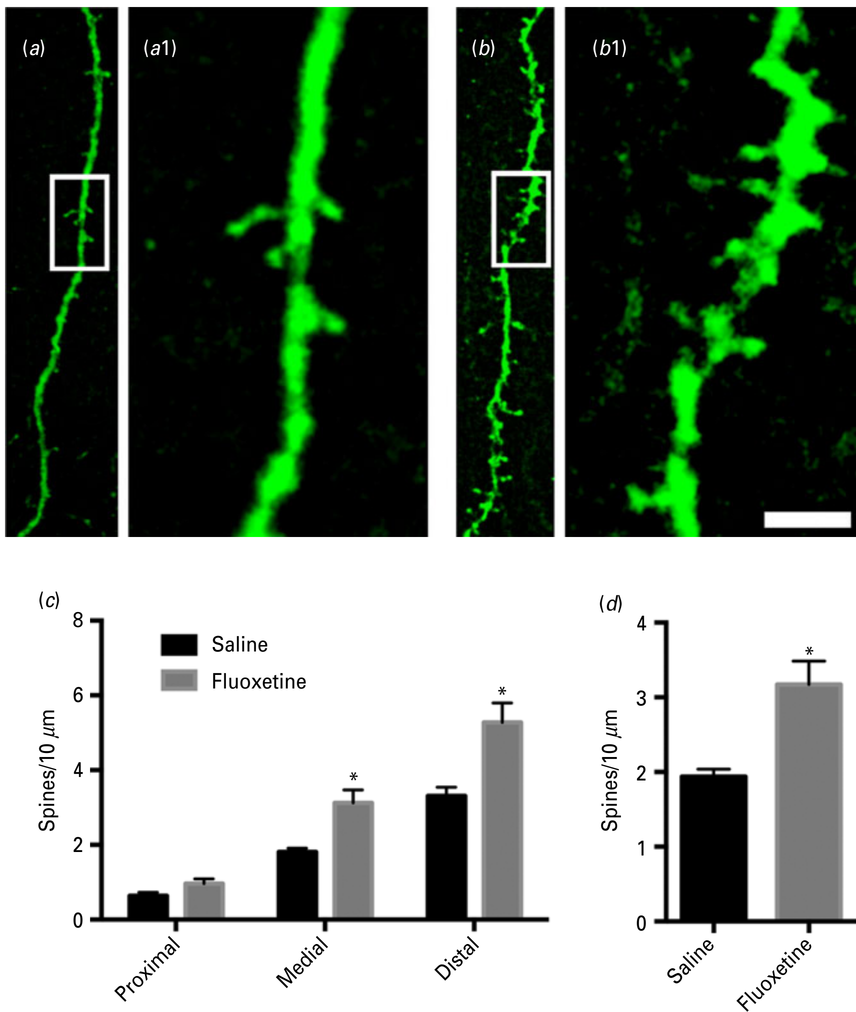

Fig. 5. Effects of fluoxetine on the spine density of interneurons in the mPFC. (*a* & *b*): Reconstruction of focal planes showing a dendrite carrying spines of an eGFP-expressing interneuron of animals treated with saline (*a*) or fluoxetine (*b*). (*a1* & *b1*): Higher magnification images showing the difference in dendritic spine density observed between both groups. (*c*): Histograms showing the dendritic spine density per 10 μm at three different segments of 60 μm each, considering their distance to the cell soma. (*d*): Graph showing the total spine density after fluoxetine treatment. (Two-way ANOVA; *p*-values *<0.05). Scale bar 8 μm in (*a*) and (*b*), 2.1 μm in (*a1* and *b1*).

expression of molecules related to inhibitory neurotransmission correspond to a compensatory mechanism.

We also confirm that this increase in the expression of molecules related to cortical inhibition in the hippocampus and mPFC is correlated with significant increases in SYN and PSA-NCAM (Varea et al., 2007a, b; Guirado et al., 2012) suggesting the formation of new inhibitory synapses after fluoxetine treatment. In fact, previous studies from our laboratory have demonstrated that PSA-NCAM is exclusively expressed by a subpopulation of interneurons in the mPFC of both humans and mice (Varea et al., 2005, 2007a). In the hippocampus PSA-NCAM is found in a subpopulation of interneurons (Nacher et al., 2002a; Gómez-Climent et al., 2010) but is also intensely expressed in the axons of a subset of granule neurons (Seki and Arai, 1999). In the rest of hippocampal structures, PSA-NCAM expression is mainly restricted to inhibitory elements (Gómez-Climent et al., 2010). Interestingly, interneurons expressing PSA-NCAM showed more reduced density of synaptic inputs, dendritic arborisation and dendritic spine density (corroborated in the present study, Fig. S2) compared to interneurons lacking this molecule (Gómez-Climent et al., 2010). However, its long-term specific ablation decreases the spine density of interneurons (Guirado et al., 2013). Indicating that an altered expression of PSA-NCAM, as that found after fluoxetine treatments (Varea et al., 2007a; Karpova et al., 2011; Guirado et al., 2012), might influence the structure of interneurons.

However, the present results suggest that PSA-NCAM may not be directly implicated in the structural changes we have described in the mPFC interneurons. The GAD67- EGFP expressing interneurons in which the structural features have been analysed normally do not show PSA-NCAM expression in their somata, neurites or in the puncta located in their projection fields in layers

I and II (Gilabert-Juan et al., 2013). Moreover, we did not find an increase in the expression of PSA-NCAM in the mPFC as we expected (only a non-significant trend). This contrast in the ability to up-regulate PSA-NCAM expression after fluoxetine treatment between hippocampal and mPFC interneurons may be due to differences in the phenotype (differential expression of calcium binding proteins and neuropeptides) of these subsets of inhibitory neurons. In fact, in control animals the interneurons that express PSA-NCAM in basal conditions belong to different phenotypic subpopulations in these two regions (Gómez-Climent et al., 2010).

Finally, we found significant reductions in the number of PV-expressing interneurons surrounded by PNN, both in the mPFC and the CA1 region of the hippocampus. This is in accordance with a previous report, which found similar reductions in the CA1 region and the basolateral amygdala (Karpova et al., 2011). The presence of PNNs is considered a marker of the maturation degree of PV-expressing interneurons and, in fact, the appearance of PNNs during late development has been used as an indication of critical period closure (Hensch, 2005; Bavelier et al., 2010; Yamada and Jinno, 2013), in which PV-expressing interneurons and their afferent connections play a critical role. In fact, removal of PNNs produces a dematuration of the axonal boutons innervating the pyramidal neurons, which results in a reopening of the critical periods (McRae et al., 2007; Nowicka et al., 2009). These results suggest that this same type of plasticity might be occurring in the mPFC and hippocampus after fluoxetine treatment. However, as mentioned above, we only found a non-significant trend for a decrease in the density of perisomatic puncta expressing PV and SYN in the mPFC and no changes in the hippocampus, suggesting that, despite its effects on PNNs, the duration of this chronic fluoxetine treatment (two weeks) is not sufficient to induce changes in the perisomatic innervation of pyramidal neurons.

Summarizing, the effects of antidepressants, affecting the structure and connectivity of certain interneuronal subpopulations, probably through the alteration in the specific expression of PSA-NCAM in interneurons and the relationship of these inhibitory cells with PNNs, may induce a window of plasticity resembling that of the critical periods. These changes may contribute in reverting the alterations of GABAergic neurotransmission in major depression patients (Sanacora et al., 2002, 2004) and, consequently, new therapeutic approaches directed to the modulation of PNNs or PSA-NCAM may constitute promising strategies to the development of innovative antidepressant drugs.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000406.

Acknowledgments

Spanish Ministry of Economy and Competitiveness BFU2012-32512, MICINN-PIM2010ERN-00577/NEU-CONNECT in the frame of ERA-NET NEURON, Generalitat Valenciana ACOMP/ 2012/229 and Prometeo Excellence Program PROMETEO2013/069 and the Fundación Alicia Koplowitz to JN. RG had a FPI pre-doctoral fellowship from the Spanish Ministry of Education and Science (BES 2007-15757). MPR had a FPU pre-doctoral fellowship from the Spanish Ministry of Economy and Competitiviness (FPU12/03200). EC is supported by the ERC (Project Number: 322742 iPlasticity), Sigrid Juselius Foundation and Academy of Finland Centre of Excellence program.

Statement of Interest

None.

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