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# **The distinct characteristics of somatostatin neurons in the human brain**

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## **Abstract**

Somatostatin cells are frequently described as a major population of GABAergic neurons in the cerebral cortex. In this study we performed a comprehensive analysis of their molecular expression, morphological features and laminar distribution. We provided a detailed description of somatostatin neurons in the human prefrontal cortex, including their proportion in the total neuron population, laminar distribution, neurotransmitter phenotype as well as their molecular and morphological characteristics using immunofluorescence and RNAscope *in situ* hybridization. We found that somatostatin neurons comprise around 7% of neocortical neurons in the human Brodmann areas 9 and 14r, without significant difference between the two regions. Somatostatin cells were NeuN positive and synthesized vesicular GABA transporter and glutamate decarboxylase 1 and 2, confirming their neuronal nature and GABAergic phenotype. Somatostatin cells in the upper cortical layers were small, had a high expression of somatostatin mRNA, a relatively low expression of somatostatin peptide and co-expressed calbindin. In the lower cortical layers, somatostatin cells were larger with complex somato-dendritic morphology, typically showed a lower expression of somatostatin mRNA and a high expression of somatostatin peptide, and co-expressed neuronal nitric oxide synthase (nNOS) and neuropeptide Y (NPY), but not calbindin. Somatostatin neurons in the white matter co-expressed MAP2. Based on their somato-dendritic morphology, cortical somatostatin neurons could be classified into at least five subtypes. The somatostatin neurons of the human prefrontal cortex show remarkable morphological and molecular complexity, which implies that they have equally complex and distinct functions in the human brain.

**Keywords:** somatostatin, prefrontal cortex, human, GABA, interneurons, schizophrenia

## Main Text

### INTRODUCTION

Somatostatin (SOM) is a peptide encoded by the *SST* gene. Its isoform somatostatin-14 is expressed in a major subset of GABAergic neurons [1, 2] and is involved in the modulation of cortical microcircuits, which is essential for regulating higher cognitive functions [3–9]. SOM<sup>+</sup> neurons play an important role in spatial working memory by selectively modulating pyramidal neurons [10]. In addition, due to their involvement in synaptic plasticity, they represent an important component in the processes of learning [10].

Reduced levels of SOM expression have been reported in several neuropsychiatric and neurodegenerative disorders, such as major depressive disorder, schizophrenia, bipolar disorder and Alzheimer's disease [10, 11] and SOM<sup>+</sup> neurons appear to play an important role in the pathogenesis these disorders. Nevertheless, the exact causes of reduced SOM expression in these disorders remain largely unknown. Most of these disorders affect the prefrontal cortex (PFC), which plays a key role in the delicate integration of relevant information from other regions of the brain. It is possible that even smaller alterations to a particular subpopulation of SOM<sup>+</sup> neurons could potentially lead to serious dysfunction of the PFC. Therefore, providing a detailed understanding the molecular organization of SOM<sup>+</sup> neurons in the human PFC is of great importance and would enable determining the intricate physiological roles of SOM<sup>+</sup> neurons. Furthermore, SOM and its analogs may have potential as future pharmacological treatments for brain disorders characterized by reduced SOM expression, which gives even more incentive to focus research on SOM<sup>+</sup> neurons [10, 11]. Nevertheless, data on the molecular characterization of SOM<sup>+</sup> neurons in the human PFC is severely lacking.

In general, the calcium-binding proteins parvalbumin (PV), calbindin (CB) and calretinin (CR) are the most common markers used to define the major populations of GABAergic neurons [12], though other molecular, morphological and electrophysiological classification criteria are also described [5, 13–15]. In most classifications, SOM immunolabelling is considered to visualize the vast majority of CB<sup>+</sup> neurons as well as additional GABAergic neurons that are not visualized by CB immunolabelling. This is why SOM is typically considered a more eligible marker that encompasses a larger subset of cortical GABAergic neurons than CB [9] – in essence CB<sup>+</sup> neurons are increasingly being regarded as merely a subpopulation of SOM<sup>+</sup> neurons and are rarely included in newer interneuron classifications, especially in rodents [15]. However, it is important to accentuate that this classification has never been explicitly confirmed in the primate brain and especially not in humans. This means that inferences on the GABAergic cortical network are often done by attempting to extrapolate data from rodents, i.e. descriptions of cortical network connectivity (and conclusions on its role in the pathogenesis of human diseases) are heavily reliant on whether the rodent model is correct for the human brain.

Based on their morphology, SOM<sup>+</sup> neurons are typically classified into two major subpopulations – Martinotti and non-Martinotti cells [16–19]. Martinotti cells have an ascending axon with synaptic targets located in layer I of the cerebral cortex, where they form significant axonal plexuses [20, 21]. There, the axons of Martinotti cells establish synapses with the terminal branches of the apical dendrites of principal cells. Unlike Martinotti cells that have a distinct axonal morphology with clearly defined synaptic targets, non-Martinotti cells are simply defined as all SOM<sup>+</sup> cells that lack significant axonal plexuses in layer I [20]. Besides their intriguing morphology, studies in rodents revealed that a portion of SOM<sup>+</sup> neurons co-express (to varying degrees) different molecular markers [22], including CR [15], CB [23, 24], neuropeptide Y (NPY) [24], cholecystokinin (CCK) [25], nNOS [16, 17, 24, 25], and substance P receptor (SPR) [18, 26].

In most species, SOM<sup>+</sup> cell bodies are present in all cortical layers, except layer I. In primates, SOM<sup>+</sup> cells have been described as less densely packed than in the rat cortex and as being located in superficial and deep bands in the cerebral cortex [27]. In the PFC of the cynomolgus monkey (*Macaca fascicularis*), SOM<sup>+</sup> cell bodies were found in layers II – III and V – VI [28]. In the human brain, their laminar distribution has been described as uneven, with some studies describing them only in layers IV – VI [29], others describing them as predominantly present in layers II and III [30], and yet others describing them in all cortical layers,

including in layer I [31, 32]. In general, previous studies on SOM<sup>+</sup> neurons in humans used different methodology (either only immunohistochemistry or only RNA *in situ* hybridization), analyzed different cortical regions (though none of them focused on the PFC) and assessed varying pathologies, while coming to somewhat conflicting findings. In order to resolve these discrepancies in literature, it is necessary to conduct systematic studies that first assess SOM<sup>+</sup> neurons in non-pathological tissue utilizing multiple complementary histological methods.

The role of SOM<sup>+</sup> neurons in the human brain is still understudied and their laminar distribution as well as morphological and molecular features in the human brain are not resolved. In particular, no studies so far addressed the molecular expression, morphological features and laminar distribution of SOM<sup>+</sup> neurons in the human PFC. Therefore, the aim of this paper is to give a comprehensive molecular and morphological characterization of SOM<sup>+</sup> neurons in phylogenetically and functionally entirely different cortical areas (Brodman areas 9 and 14r) of the human PFC using novel histological methodology combining immunofluorescence and RNAscope *in situ* hybridization.

## MATERIALS AND METHODS

### Brain tissue samples

The brain samples of five male human subjects aged from 29 to 51 years with a postmortem delay of 6 to 11 hours were analyzed in this study (Table S1).

The subjects had no medical history of neurological or psychiatric disorders and no neuropathological deviations in the brain on autopsy. Relevant medical history was obtained from both autopsy reports and medical records. All analyzed subjects died without preagonal state, and the postmortem delay represents the actual interval of neuron death. The brain tissue is a part of the Zagreb Neuroembryological Collection. It was obtained with the approval of the Ethics Committee of Zagreb University School of Medicine (380-59-10106-14-55/152). The information on the subject's identity and history is stored in secure records, and the brain tissue is given a code indicating only the subject's age.

The brain tissue was cut into blocks following Talairach's coordinates [33]. Tissue blocks of the dorsolateral and ventral prefrontal cortex (dlPFC and vPFC) containing the superior frontal gyrus (Brodmann area 9, BA9) and straight gyrus (Brodmann area 14r, BA14r) (Figure S1) respectively were selected for double labelling immunofluorescence [34–36]. BA9 and BA14r were delineated on NeuN immunolabelled sections according to relevant literature [37–39]. Cortical layers were also distinguished using NeuN immunolabelled sections.

The tissue was first fixed by immersion in 4% paraformaldehyde for 24 h, then dehydrated in an ethanol cascade (70%, 96%, 100%), embedded in paraffin, kept in toluene [40] and finally cut on a microtome into 20 µm thick coronal slices [41] and mounted on VitroGnost Plus Ultra adhesive microscope slides (BioGnost, Zagreb, Croatia).

### Double labelling immunofluorescence

Double labelling immunofluorescence was performed according to standard protocols for paraffin-embedded tissue [42]. Histological sections were first photobleached for 48 h using a LED light source [43, 44] in order to reduce autofluorescence. The sections were deparaffinized and heat antigen retrieval was performed in citrate-based (pH 6.0) unmasking solution [45]. Next, protein blocking was done by incubating the sections for 1 h at room temperature (RT) in normal donkey serum (NDS; Chemicon, USA) diluted 1:30 in permeabilization solution (0.3% Triton X-100 in 1X PBS; Sigma-Aldrich, USA). Afterwards the sections were incubated overnight in primary antibodies (Table S2) at 4°C and in secondary antibodies (Table S2) for 2 h at RT. The sections were treated with TrueBlack® Lipofuscin Autofluorescence Quencher (Biotium, USA) to further reduce autofluorescence [46] and coverslipped with VECTASHIELD® Antifade Mounting Medium (Vector Laboratories, USA).

Somatostatin antibodies were used in combination with all other primary antibodies listed in Table S2. Furthermore, the calbindin antibody was used in combination with the mouse monoclonal NeuN antibody.

### RNAscope *in situ* hybridization

The expression of mRNA in the tissue samples was evaluated using RNAscope *in situ* hybridization (Advanced Cell Diagnostics, USA). The RNAscope® Fluorescent Multiplex Assay (Advanced Cell Diagnostics) was used to detect multiple target mRNAs. A standard protocol, according to the manufacturer's instructions (Advanced Cell Diagnostics) was used [47–49]. Pretreatment included deparaffinization, target retrieval with Target Retrieval Reagent, and incubation in Protease Plus reagent (Advanced Cell Diagnostics). The slides were then incubated in the appropriate Probe Mix reagent (Table S3) followed by successive amplifier (AMP) reagents (Advanced Cell Diagnostics). Depending on the probes used, visualization was done using channel appropriate dyes (TSA™ Plus Fluorescein, TSA™ Plus Cyanine 3 System and/or TSA™ Plus Cyanine 5 System; Akoya Biosciences, Marlborough, MA, USA).

After RNAscope *in situ* hybridization was completed, the same slides were further processed for single labelling immunofluorescence, according to the previously described protocol (starting with the protein blocking step).

Histological sections were imaged using a laser confocal microscope (Olympus FLUOVIEW FV3000RS, Japan) on high power magnification and using Z-stack in order to visualize the entire section thickness.

### Quantification of immunoreactive cells

Quantification of immunoreactive cells was done using NeuroLucida 2020 (MBF Bioscience, Williston, Vermont, USA) and NeuroLucida Explorer (MBF Bioscience) on confocal images of double labelled immunofluorescent histological slides (NeuN/SOM and NeuN/CB immunolabeling). NeuN immunolabeling was used to delineate the cortical layers in NeuroLucida. During analysis, layer I was further divided into layer Ia (superficial part of layer I) and layer Ib (deep part of layers I). From each brain specimen (n = 5) at least three slides were analyzed using the *Detect cells* function in NeuroLucida software.

The results of the quantification are presented as: a) the number of immunolabelled neurons per contour surface (further: surface density) and b) the proportion of SOM<sup>+</sup> and CB<sup>+</sup> neurons within the total cortical neuron population (defined as the total number of NeuN immunolabelled cells).

### Morphometric analysis

Morphometric analysis of SOM<sup>+</sup> cells was done using NeuroLucida 2020 and NeuroLucida Explorer. The cell bodies of SOM<sup>+</sup> cells were reconstructed on confocal images using the following criteria: the cell body was visible in its entirety and the most proximal parts of the cell's processes could be identified. A total of 552 SOM<sup>+</sup> cell bodies were chosen for reconstruction (278 from BA9 and 274 from BA14r). The following morphometric parameters were analyzed: *Area*, *Aspect ratio* and *Roundness*. The analyzed morphometric parameters are defined in NeuroLucida Explorer as follows:

- a) *Area* – the two-dimensional cross-sectional surface contained within the boundary of the cell body,
- b) *Aspect ratio* – the ratio between the minimum and maximum diameter of the cell body that indicates the degree of flatness and has a value between 0 and 1,
- c) *Roundness* – the square of *Compactness*, which is calculated as  $\frac{\sqrt{\frac{4}{\pi} \times Area}}{\text{maximum diameter}}$ ; *Roundness* has a value between 0 and 1.

### Statistical analysis

The statistical analysis was done using GraphPad Prism, version 9.2.0 (GraphPad Software, La Jolla, USA).

Data referring to different brains (proportions and surface density) as well as morphometric parameters for which a normal distribution was determined to be more likely (*Aspect ratio* and *Roundness*) are shown as arithmetic mean  $\pm$  standard deviation (SD).

Morphometric parameters for which a lognormal distribution was determined to be more likely (*Area*) are shown as geometric mean  $\times$  geometric standard deviation factor (GSDF).

In subsequent statistical analysis, data from the same brain were paired. A *P*-value of less than 0.05 was considered to be statistically significant for all statistical tests.

The paired t-test was used to test the differences between the proportions and surface densities of somatostatin neurons between BA9 and BA14r.

Welch's t-test was used to test the differences in *Roundness* and *Aspect ratio* between supragranular and infragranular layer somatostatin neurons. Mann Whitney's test was used to test the differences in cell body *Area*.

## RESULTS

There were no observable qualitative differences between Brodmann area 9 (BA9) and Brodmann area 14r (BA14r), and quantitative analysis confirmed no significant differences between these two regions (Figure S2). Therefore, the results represent pooled data from both regions of the PFC, unless stated otherwise.

### **SOM<sup>+</sup> cells are GABAergic neurons present in cortical layers II – VI as well as in the white matter (WM)**

SOM<sup>+</sup> cells in all cortical layers co-expressed NeuN (Figures 1A and 1B). Both immunofluorescence and RNA *in situ* hybridization confirmed that somatostatin neurons were present in cortical layers II – VI as well as in the superficial white matter (WM) (Figure 1C). On average, SOM<sup>+</sup> neurons represented  $6.83 \pm 1.46\%$  of the total neuron population. Somatostatin fibers were generally highly immunoreactive and present in all cortical layers and the superficial white matter (Figures 1D and 1E), but were most abundant in layers I, IV and VI.

While the surface density of SOM<sup>+</sup> neurons was almost twice as high in layers II and IV compared to other cortical layers (Figure 2A), their proportion within the total neuron population was mostly uniformly distributed across cortical layers II – VI (Figure 2B).

RNA *in situ* hybridization confirmed the GABAergic neurotransmitter phenotype of SOM<sup>+</sup> neurons as they expressed *SLC32A1* (VGAT) as well as *GAD1* (GAD67) and *GAD2* (GAD65) mRNA, but did not express *SLC17A7* (VGLUT1) mRNA (Figures 2C and 2D).

SOM<sup>+</sup> neurons were also present in the superficial white matter, where they were more abundant than any other analyzed interneuron population (CR, PV or CB).

### **CB<sup>+</sup> cells are GABAergic neurons present predominantly in supragranular cortical layers**

In contrast to SOM<sup>+</sup> cells, CB<sup>+</sup> cells were significantly more abundant in supragranular than infragranular layers (Figure 2E). As with SOM<sup>+</sup> cells, all CB<sup>+</sup> interneurons co-expressed NeuN as well as the GABAergic markers *SLC32A1*, *GAD1* and *GAD2*. CB<sup>+</sup> neurons were only sporadically present in the deep parts of layer I.

### **SOM<sup>+</sup> neurons co-express CB predominantly in supragranular cortical layers**

Double labelling immunofluorescence revealed that in layers V and VI and the WM, SOM<sup>+</sup> and CB<sup>+</sup> neurons represented two almost separate neuronal populations, while in layers II and III almost all cells that expressed SOM co-expressed CB (Figures 2F, 3A and 3B). Overall, throughout all cortical layers, 67.73% of CB<sup>+</sup> interneurons co-expressed SOM, and 49.94% of SOM<sup>+</sup> neurons co-expressed CB.

SOM/CB double labelling revealed three neuron subpopulations with characteristic laminar distributions. SOM<sup>+</sup>/CB<sup>+</sup> cells were the most abundant in layers II and III, SOM<sup>+</sup>/CB<sup>-</sup> cells being most abundant in layers IV – VI, and SOM<sup>-</sup>/CB<sup>+</sup> cells being most abundant in layers II and VI (Figure 3C).

Co-expression of SOM and CB in the supragranular layers was also confirmed using RNA *in situ* hybridization (Figure 3D).

### **The expression of *SST* mRNA and SOM peptide differs between supragranular and infragranular layer neurons**

Evaluation of somatostatin mRNA and peptide (using RNA *in situ* hybridization and immunofluorescence) revealed that layer II and III SOM<sup>+</sup> neurons typically had a very high expression of *SST* mRNA, but a comparatively lower expression of SOM peptide (Figure 3E). The reverse was true for layer V and VI as well as the WM, where SOM<sup>+</sup> neurons had a low to medium expression of *SST* mRNA, but a comparatively higher expression of SOM peptide (Figure 3E). These findings were consistent in all analyzed subjects and when using different combination of markers. Occasionally, individual cells with both high *SST* mRNA and high peptide expression were observed (Figure 3F).



### **There are morphological differences between supragranular and infragranular SOM<sup>+</sup> neurons**

Morphological analysis revealed that most SOM<sup>+</sup> neurons could be classified into one of five different types based on their size, cell body shape and laminar position (for a detailed overview see Table 1 and Figures 4A and 4B). The large fusiform (type 4) and pleomorphic (type 5) SOM<sup>+</sup> neurons found predominantly in the infragranular layers and white matter were particularly notable.

The axons of a substantial portion of SOM<sup>+</sup> neurons likely arborized in layer I where somatostatin fibers were particularly abundant.

The differences in morphometric parameters between supragranular and infragranular layers SOM<sup>+</sup> neurons were particularly pronounced and statistically significant ( $P < 0.0001$  for all analyzed morphometric parameters) (Figures 4C, 4D and 4E) confirming that supragranular and infragranular SOM<sup>+</sup> neurons differ not only in their molecular expression, but also in their morphological properties.

### **Some SOM<sup>+</sup> neurons co-express specific molecular markers**

Further double labelling immunofluorescence was performed to better characterize the different morphological types of SOM<sup>+</sup> neurons. These experiments revealed that almost all infragranular layer and WM SOM<sup>+</sup> neurons co-expressed nNOS (Figure 5A), unlike in supragranular layers where the level of co-expression was low. SOM<sup>+</sup> neurons in the WM also frequently co-expressed the neuronal marker MAP2 (Figure 5B), however, practically no MAP2 co-expression was observed in supragranular and infragranular cortical layers. Furthermore, large SOM<sup>+</sup> neurons (types 4 and 5) were found to co-express NPY (Figure 5C and Figure S3). Overall, throughout all cortical layers, all NPY<sup>+</sup> cells co-expressed SOM, and 10.28% of SOM<sup>+</sup> cells co-expressed NPY. The level of co-expression was higher in infragranular layers and the WM than in supragranular layers (Figure 5D). No co-expression was observed between SOM and the interneuron markers CR (Figures 6A and 6B) and PV (Figure 6C), nor between CB and CR or PV (Figure S4). SOM<sup>+</sup> neurons never co-expressed the molecular marker SMI-32 (Figure 6D)..

## DISCUSSION

In this study, we provided a detailed description of SOM<sup>+</sup> GABAergic neurons in the human PFC, including their proportion in the total neuron population, laminar distribution, neurotransmitter phenotype as well as their molecular and morphological characteristics.

### **The laminar distribution of SOM<sup>+</sup> neurons is uniform across ventral and dorsal areas of the human PFC**

One of the most relevant findings of our study is the uniform laminar distribution of SOM<sup>+</sup> neurons in the human PFC. The major issue when addressing the laminar distribution of any cell population seems to be the lack of consensus on how to report laminar distributions [27, 29–32]. The surface density of SOM<sup>+</sup> neurons is almost twice as high in layer II and IV than in other layers, while the proportions of SOM<sup>+</sup> neurons in these layers are only slightly higher. This indicates that the high density/number of SOM<sup>+</sup> neurons in layers II and IV is mostly due to the high neuronal densities of these layers in general, and not due to a disproportionate increase of SOM<sup>+</sup> neurons in these layers. Indeed, the average proportions of SOM<sup>+</sup> neurons vary rather little between cortical layers, with the notable exception of layer I, where SOM<sup>+</sup> neurons are present only sporadically.

Quantification of SOM<sup>+</sup> neurons with low immunoreactivity is methodologically challenging and is most reliably done using double labelling with NeuN, CB or *SST* mRNA.

Previous studies on SOM<sup>+</sup> neurons in the human brain differed vastly in methodology, pathology and cortical regions analyzed [29–32] and none analyzed the PFC in normotypical postmortem brain tissue. Some of these studies came to conflicting findings using immunohistochemistry, with González-Albo et al. [30] concluding that SOM<sup>+</sup> neurons were more prevalent in layers II – III and Zhu et al. [29] stating that they were more prevalent in layers IV – VI. In addition, the study by Zhu et al. [29] found that SOM<sup>+</sup> neurons constituted only half the proportion we determined for the PFC. Regional differences as well as the fact that Zhu et al. used intraoperative tissue of epileptic patients might explain the different findings.

### **Specific co-expression of SOM and other molecular markers redefines the human GABAergic network**

Another major finding in this study is a detailed molecular redefinition of the somatostatin subpopulation in the human brain. We noted a significant difference in the level of colocalization between SOM and CB in the human PFC. This is particularly evident when comparing supragranular with infragranular layers, with almost all of the double labeled cells being located in layers II and III, and it was confirmed both by immunofluorescence and RNAscope *in situ* hybridization. Such a difference in co-expression between upper and lower cortical layers was previously not described in humans, and contrasts with findings in rodents, especially rats where the level of co-expression seems to be the highest in layers V and VI [16, 23, 24]. The study by González-Albo et al. [30] also indicated that CB<sup>+</sup> interneurons were about 50% more numerous than SOM<sup>+</sup> neurons, though detailed quantification seems to have only been done in layers II and III. The differences in our findings might also arise from the fact that González-Albo et al. [30] identified relatively few SOM<sup>+</sup> cells in layers V and VI (see Figure 4 from González-Albo et al., 2001) whereas in our study SOM<sup>+</sup> cells were numerous in these layers. As previously mentioned, this could be due to regional differences between different parts of the cortex or methodological differences between the studies.

Furthermore, we clearly demonstrated that, in the human PFC, interneurons that express either SOM or CB represent a neuron population that does not overlap with either CR or PV, which is distinct from the findings in rodents [15, 16, 18, 23, 24]. In addition, this neuron population based on SOM and CB expression can be divided into three subpopulations: SOM<sup>+</sup>/CB<sup>+</sup> cells, SOM<sup>+</sup>/CB<sup>-</sup> cells and SOM<sup>-</sup>/CB<sup>+</sup> cells. All of the aforementioned provides important evidence that the SOM network of the human PFC differs fundamentally from what has previously been described in rodents. In particular, the finding that SOM<sup>+</sup> and CB<sup>+</sup> neurons are only partially overlapping GABAergic neuron populations challenges the notion that CB could be replaced as an important interneuron marker in the human brain and calls for a reevaluation of the applicability of rodent interneuron classifications in the human PFC.

## **Somatostatin neurons of different layers have different molecular and morphological profiles with important functional implications within cortical neural circuits**

In this study we demonstrated that there are at least five morphological types of somatostatin neurons in the human PFC, each of which has characteristic immunoreactivity, cell body shape and size, and even laminar distribution (Table 1). The fact that certain morphological cell types are largely constrained to certain cortical layers suggests that they may also have specific functional properties [13, 50, 51]. In particular, the type 3 cells found predominantly in layer IV and the most superficial part of layer V might correspond to the non-Martinotti cells targeting layer IV PV<sup>+</sup> neurons described in rodents, whereas the type 1 cells found in layers II – VI most likely correspond to the Martinotti cells whose synaptic targets are the apical dendrites in layer I.

The morphological types 4 and 5 are particularly interesting since they are very large cells present predominantly in layers V and VI as well as the white matter. So far, these cell types have received little attention in most research on somatostatin neurons, even though their morphology, size and molecular profile are vastly different from the other types of somatostatin neurons. We determined that most of these cells co-express NPY and nNOS, but do not co-express CB. Interestingly, NPY in the human PFC was not co-expressed by the smaller SOM<sup>+</sup> neuron types (1, 2 and 3) and was overall co-expressed in only around 10% of SOM<sup>+</sup> neurons, which is an interesting contrast to the high levels of co-expression (up to 80%) determined in the hippocampus [52], the rat striatum [53], and the rat barrel cortex [54]. Nevertheless, our findings are mostly in line with the study by Raghanti et al. (2013), which demonstrated that NPY<sup>+</sup> neurons represent a relatively small subpopulation of cortical neurons in the primate BA22 [55], though the aforementioned study did not evaluate the level of co-expression of NPY and SOM. It should also be accentuated that all NPY<sup>+</sup> cortical neurons we observed co-expressed SOM, which suggests that NPY<sup>+</sup> neurons could be a subpopulation of the larger SOM<sup>+</sup> neuron population.

We further investigated the large SOM<sup>+</sup> neurons in the WM, because they presented a rather prominent neuron population in the WM. Double labelling revealed that SOM<sup>+</sup> neurons in the WM co-expressed nNOS, a marker often ascribed to long-range GABAergic connections [56]. The SOM<sup>+</sup> neurons found in the white matter expressed exclusively GABAergic molecular markers (VGAT, GAD65 and GAD67), and also co-expressed MAP2 – a marker commonly found in projection neurons in the adult brain (but not SMI-32, another marker typical for pyramidal projection neurons). These neurons are, therefore, very intriguing since they could represent long-projecting GABAergic cells.

Overall, SOM<sup>+</sup> neurons in the human PFC have different morphological and molecular characteristics in different cortical layers, which is particularly pronounced between supragranular and infragranular layers (Table 1).

### **Conclusion**

In conclusion, SOM<sup>+</sup> neurons represent a relatively large population of GABAergic neurons in the human PFC, constituting on average 6.83% of the total neuron population. They are found in cortical layers II – VI as well as in the white matter, and their molecular and morphological characteristics differ between cortical layers, particularly between supragranular and infragranular layers. Our findings challenge the notion that data on SOM<sup>+</sup> neurons can always be reliably extrapolated from rodents to humans and give reason to shift focus from models reliant solely on rodents to models incorporating studies of the human brain. In particular, understanding the characteristics and function of SOM<sup>+</sup> neurons in the human PFC is of great importance for determining the possible effects of their alterations in diseases affecting the central nervous system, especially psychiatric disorders such as major depressive disorder, schizophrenia, and bipolar disorder.

## **Statements and Declarations**

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### **Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

### **Author Contributions**

Ivan Banovac and Dora Sedmak designed the study. Ivan Banovac acquired the data. All authors analyzed the data, drafted the manuscript and performed a critical revision of the manuscript.

### **Data Availability**

All data presented in this study are available in the article and accompanying supplementary information. Raw data are available on request from the corresponding author.

### **Ethics approval**

This study was approved by the Ethics Committee of Zagreb University School of Medicine (380-59-10106-14-55/152).

### **Consent to participate**

Not applicable.

### **Consent to publish**

Not applicable.

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## Figure and Table Legends

**Figure 1.** Laminar distribution of SOM<sup>+</sup> cells in the human PFC. (A) SOM/NeuN double labeling showing that all SOM<sup>+</sup> cells co-express NeuN. Scale bar: 200  $\mu$ m. (B) High-power confocal images showing NeuN and SOM co-expression in supragranular (SG) and infragranular cortical layers. Scale bar: 20  $\mu$ m. (C) Dot plot showing laminar distribution of SOM<sup>+</sup> neurons. (D) Heat map showing relative quantities of SOM<sup>+</sup> fibers in different cortical layers. (E) Binary confocal image of SOM immunolabeling in human BA14r. Scale bar: 200  $\mu$ m.

**Figure 2.** Laminar distribution of SOM<sup>+</sup> and CB<sup>+</sup> cells and GABAergic phenotype of SOM<sup>+</sup> cells in the human PFC. (A) Surface density of SOM<sup>+</sup> neurons in different cortical layers. Data presented as mean  $\pm$  SD. (B) Proportion of SOM<sup>+</sup> neurons in different cortical layers. Data presented as mean  $\pm$  SD. (C) SOM/*SLC32A1* (VGAT)/*SLC17A7* (VGLUT1) triple labeling showing a SOM<sup>+</sup> neuron co-expressing only *SLC32A1* (VGAT) mRNA. Scale bar: 20  $\mu$ m. (D) SOM/*GAD1*/*GAD2* triple labeling showing a SOM<sup>+</sup> neuron co-expressing both *GAD1* and *GAD2* mRNA. Scale bar: 20  $\mu$ m. (E) Proportion of CB<sup>+</sup> neurons in different cortical layers of BA9 and BA14r. Data presented as mean  $\pm$  SD. (F) Proportions of SOM<sup>+</sup> cells co-expressing CB and CB<sup>+</sup> cells co-expressing SOM in different cortical layers.

**Figure 3.** Differences in molecular expression between supragranular and infragranular layer SOM<sup>+</sup> cells in the human PFC. (A) Confocal acquisition of CB/SOM double labeling in the human BA9. Scale bar: 200  $\mu$ m. (B) High power confocal image showing CB and SOM co-expression in supragranular cortical layers. Scale bar: 20  $\mu$ m. (C) Dot plots showing laminar distributions of CB<sup>+</sup>/SOM<sup>-</sup>, CB<sup>+</sup>/SOM<sup>+</sup> and CB<sup>-</sup>/SOM<sup>+</sup> cells. (D) CB/*SST* double labeling showing CB<sup>+</sup> neurons co-expressing *SST* mRNA in human BA14r. Scale bar: 20  $\mu$ m. (E) SOM/*SST* double labeling showing differences between SOM peptide and *SST* mRNA expression in supragranular (SG) and infragranular (IG) layers of human BA14r. Scale bar: 10  $\mu$ m. (F) SOM/*SST* double labeling showing a sporadically present SOM<sup>+</sup> neuron in human BA9 with both high SOM peptide and *SST* mRNA expression. Scale bar: 20  $\mu$ m.

**Figure 4.** Morphological analysis of SOM<sup>+</sup> cells in the human PFC. (A) SOM immunolabeling showing the five morphological types of SOM<sup>+</sup> neurons in different cortical layers. Data presented as mean  $\pm$  SD. (B) NeuroLucida reconstructions of representative SOM<sup>+</sup> neurons for each morphological type. Scale bar: 20  $\mu$ m. (C) *Area* (geometric mean  $\times$  GSD; logarithmic axis), (D) *Aspect ratio* (mean  $\pm$  SD), and (E) *Roundness* (mean  $\pm$  SD) of SOM<sup>+</sup> cell bodies in different cortical layers.

**Figure 5.** Molecular co-expression of SOM and other molecular markers in the human PFC. (A) SOM/nNOS double labeling showing high level of SOM/nNOS co-expression (\*) in the infragranular layers of human BA9. Scale bar: 40  $\mu$ m. (B) SOM/MAP2 double labeling showing SOM/MAP2 co-expression (\*) in the white matter of human BA14r. Scale bar: 40  $\mu$ m. (C) SOM/NPY double labelling showing SOM/NPY co-expression (\*) in a large SOM cell in the human BA14r. Scale bar: 20  $\mu$ m. (D) Proportions of SOM<sup>+</sup> cells co-expressing NPY in different cortical layers.

**Figure 6.** Molecular co-expression of SOM and other interneuron and projection markers in the human PFC. (A) Dot plot showing distribution of SOM (red) and CR (green) neurons in different cortical layers. (B) SOM/CR and (C) SOM/PV double labeling showing that that SOM<sup>+</sup> interneurons do not overlap with the CR and PV interneuron populations. (D) SMI-32/SOM double labeling showing that SOM<sup>+</sup> neurons do not co-express SMI-32. Scale bars for all panels: 200  $\mu$ m.

**Table 1.** Morphological types of SOM<sup>+</sup> neurons in the human PFC and laminar diversity of SOM<sup>+</sup> neurons.



## Tables

Table 1.

<b>Morphological types of SOM<sup>+</sup> neurons</b>				
<b>Morphological type</b>	<b>Immunoreactivity</b>	<b>Cell body size</b>	<b>Cell body shape</b>	<b>Laminar distribution</b>
1	medium	medium	slightly elongated, oval	layers II – VI
2	low	small	circular	layer II (predominantly) and layer III
3	low	very small	circular	layer IV (almost exclusively), layer V (occasionally only in the most superficial parts)
4	high	very large	extremely elongated	layers V – VI and superficial WM (predominantly), layer III (sporadically)
5	high	large	pleomorphic	layers V – VI and superficial WM
<b>Laminar diversity of SOM<sup>+</sup> neurons</b>				
<b>Cortical layer</b>	<b>Morphological cell type</b>	<b>Co-expression of other molecular markers</b>	<b>Immunoreactivity and SST mRNA expression</b>	
I	predominantly nerve fibers, cell bodies only sporadically found	CB (sporadically)	-	
II	predominantly type 2, type 1 also present	CB (high)	low – medium immunoreactivity; high mRNA expression	
III	predominantly types 1 and 2, type 4 sporadically found	CB (high), NPY (sporadically)		
IV	predominantly type 3, type 1 also present	CB (low – medium)	low immunoreactivity; medium mRNA expression	
V	predominantly types 1 and 4, types 3 (at the border with layer IV) and 5 also present	nNOS (high), NPY (medium)	high immunoreactivity; low – medium mRNA expression	
VI	predominantly types 1, 4 and 5	nNOS (high), NPY (medium)		
WM	exclusively types 4 and 5	nNOS (high), NPY (high), MAP2 (medium – high)		