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Region- and Sex-Specific Differences of Cerebral Parvalbumin Distribution and Expression in Rats.

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Parvalbumin-expressing interneurons in the brain play key roles in the modulation of various neuronal communications and their dysfunction is implicated in multiple neurological disorders. Understanding their anatomical distribution across the brain and potential sex-specific differences holds significance in neuroscience. Here we used immunohistochemistry methods and digital image analysis to evaluate parvalbumin distribution and expression across selected brain regions. The study showed a higher number of parvalbumin-positive cells in the cerebral cortex (frontal and parietal) compared to subcortical regions (midbrain, thalamus, hippocampus, striatum). However, parvalbumin immunoreactivity was similar across regions examined except the striatum. Furthermore, we observed a significant sex-specific difference in the number of parvalbumin-positive cells only in the parietal cortex. The study thus suggests strong parvalbumin expressions across the brain even in regions with a smaller number of parvalbumin-positive cells, indicative of extensive parvalbumin neuronal connections. Additionally, the study notes the potential effect of sexual dimorphism in parvalbumin distribution.

Key words: Parvalbumin; Immunoreactivity; Cerebrum; Sex dimorphism; Rats

Introduction

In the brain, many activities are influenced by intracellular calcium (Ca) changes; these include neuronal excitation, and synaptic modulation, amongst others. Parvalbumin is a Ca-binding protein which plays an important role in the regulation of intracellular Ca concentrations [2,11]. A group of interneurons express parvalbumin. Key characteristics of this class of interneurons are fast responses and effective inhibition of neighbouring primary neurons [10]. These parvalbumin-expressing neurons are mostly GABAergic interneurons and have been reported to perform vital functions in neuronal

communications [18]. For example, parvalbumin interneurons in the parahippocampal domains are key modulators of the characteristic grid cells in the medial entorhinal cortex, where they serve as how the primary neurons communicate [3, 17]. Similar inhibitory connectedness has also been reported in the lateral entorhinal cortex, where the inhibitory interneurons are involved in tuning of primary neurons [19, 24].

Obviously, parvalbumin interneurons are hugely important across the brain, as their dysfunction is implicated in a variety of brain disorders including neurodegenerative diseases, neuropsychiatric and developmental disorders [5, 18]. Disturbances in parvalbumin interneurons are reported to be a hallmark of the progression of schizophrenia, where such disruptions trigger dopaminergic and glutamatergic dysfunctions [7, 9]. Additionally, the reduction of GABAergic interneurons that have been reported in the hippocampus of human schizophrenia patients has been linked to lowered parvalbumin expression [30]. Similarly, decreased parvalbumin expression and loss of parvalbumin interneurons have been reported in transgenic mice models of Alzheimer's diseases [29]. Furthermore, the downregulation of parvalbumin in interneurons has been indicated to trigger autistic phenotypes in rodent models [6, 16]. Interestingly, parvalbumin expression in the brain may be sexually dimorphic, and such sex-specific differences may provide vital insights into disorders in which parvalbumin disruptions are implicated. Curiously, an earlier report has shown a greater deficit in the relative density of hippocampal parvalbumin-positive neurons in male schizophrenic patients compared to their female counterparts [30].

Given the hugely important role of parvalbumin interneurons in various brain functions and implications in a wide variety of brain disorders, understanding their anatomical distribution in the brain, as well potential variabilities across the sexes, will prove hugely useful resources and of high interest in the neurosciences. Hence, the present study utilized immunohistochemical methods to evaluate parvalbumin distribution and expression across selected brain regions, as well as evaluating potential sexual dimorphic differences in brain parvalbumin expression.

Materials and Methods

Animals

Adult Sprague Dawley rats of both sexes [n = 6/sex; average weight: males = 339 ± 4.84 g, females = 215 ± 4.59 g], aged 10 weeks were procured from Charles Rivers Laboratories, USA, for the study. All animal experimental protocols were performed in strict accordance to the guidelines of the National Institute of Health for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee. Animals were kept under 12-hour light-dark cycle with free access to regular food and water. Three weeks post-procurement, rats were euthanized via isoflurane inhalation, brains were rapidly excised, fixed by immersion in 10% neutral buffered formalin and processed for immunohistochemistry.

Immunohistochemistry

Brain tissues were processed for routine paraffin wax embedding. Using The Rat Brain Atlas [22] as guide, serial mid-sagittal (2-3 mm lateral to midline) sections of 5 µm thin sections were obtained. Immunohistochemistry followed protocols we have

previously established [13,21]. Sections were deparaffinized and heat mediated antigen retrieval performed in citrate-based antigen unmasking solution, pH 6.0 (Vector®, USA; #H3300). Sections were then treated for 10 min in hydrogen peroxide solution (0.3% in Tris Buffered Saline) for endogenous peroxidase blocking. Protein block was performed for 30 min in 2.5% normal horse serum (Vector® #MP-7401). Sections were then incubated in anti-parvalbumin (Novus Biologicals, USA; NB120-11427) at 1:1000 dilution, for 1 h at room temperature. Secondary incubation was performed using ImmPRESS™ (Peroxidase) Polymer Anti-Rabbit IgG Reagent (Vector® #MP-7401). DAB Peroxidase (HRP) Substrate Kit (Vector® #SK-4100) was used to reveal antibody immunoreactivity, after which sections were counter-stained in haematoxylin.

Imaging and Quantification

Digital imaging was performed using the Pannoramic 250 Flash II slide scanner (3D Histech, Budapest, Hungary) at 20x objective. Random non-overlapping images were obtained at x200 magnification (area size = $\approx 2.5 \times 10^5$ μm^2) from each animal. Ten random non-overlapping images were obtained from the frontal cortex, parietal cortex, midbrain region, thalamus and striatum. Four to five images were obtained from hippocampal CA3 region, and 5-7 from the hippocampal dentate gyrus (DG). Quantification of immunostained images were performed using the Image Analysis and Processing for Java (ImageJ), a public domain software sponsored by the National Institute of Health (USA). To quantify number of parvalbumin positive cells, Image J Cell Counter tool was used to identify and count positive cells as previously established [12]. To determine immunoreactivity of parvalbumin expression, images were analysed using the ImmunoRatio plugin on Image J, which separates and quantifies the percentage of DAB (positive immunoreactivity) by digital colour deconvolution [4]. Data for subsequent statistical analysis were averages obtained from each animal.

Statistics

Data are presented as mean ± SEM. Data were analysed on GraphPad Prism 8 software (GraphPad Inc, USA) by two-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* tests. Significant difference between sexes were further confirmed by t-test. Values of $p < 0.05$ were considered statistically significant.

Results

Immunostaining for parvalbumin shows marked expression across many brain regions in both male and female rats. Specifically, parvalbumin distribution across the cerebrum is most prominent in the cortex compared to subcortical regions (**Fig. 1** and **Fig. 2**).

Further analysis to quantify number of parvalbumin cells across selected brain regions was performed with Cell counter tool in Image J software. Two-ANOVA analysis of data obtained revealed no significant interaction ($p = 0.3357$; $F_{(6,69)} = 1.16$) between sex and brain regions. However, analysis revealed significant sex ($p < 0.01$; $F_{(1,69)} = 4.78$) and region ($p < 0.001$; $F_{(6,69)} = 19.94$) specific effects. Further Bonferroni's post-hoc test showed significantly higher number of parvalbumin cells in frontal cortex of males compared to other regions examined (parietal cortex, midbrain, thalamus, hippocampus (CA3 and DG), and striatum). Similarly for females, number

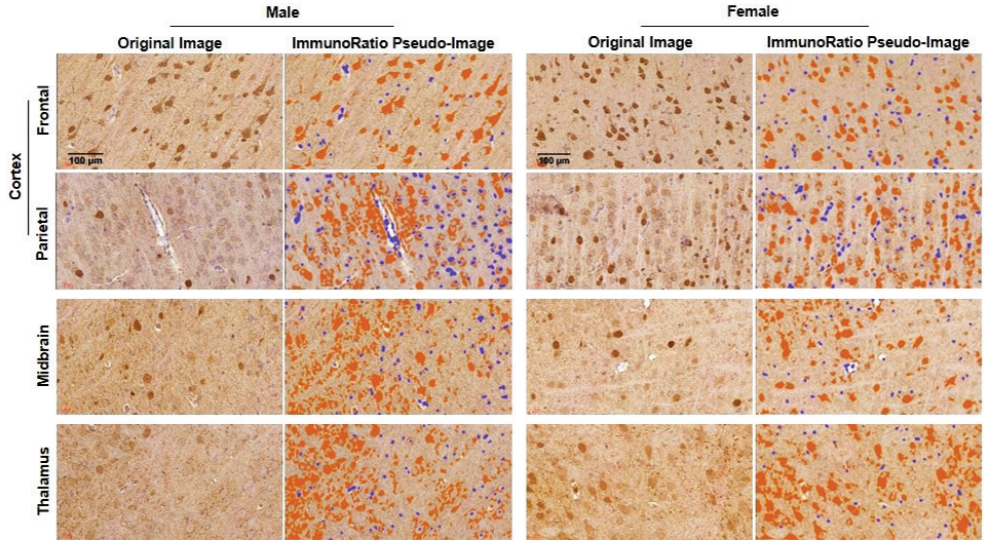

Fig. 1. Representative photomicrographs of parvalbumin expression and positive cells in the cortex (frontal and parietal), midbrain, and thalamus of male and female rats. Original images are accompanied by Pseudo-Image generated with ImmunoRatio plugin in Image J software.

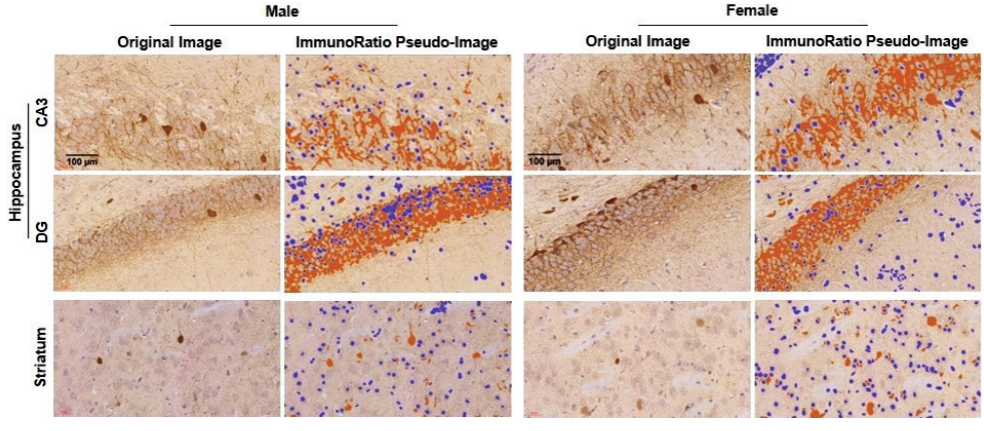

Fig. 2. Representative photomicrographs of parvalbumin expression and positive cells in the hippocampus (CA3 and DG), and striatum of male and female rats. Original images are accompanied by Pseudo-Image generated with ImmunoRatio plugin in Image J software.

of parvalbumin cells in the frontal cortex was significantly higher compared to other regions except for the parietal cortex which showed no significant difference. However, the parietal cortex had significantly higher number of parvalbumin cells compared to the hippocampus CA3 and DG, and the striatum. Bonferroni's post-hoc test showed no difference between sex for all regions, however further confirmation using t-test

revealed that in the parietal cortex, the number of parvalbumin cells is significantly higher ($p < 0.05$) compared to males (**Fig. 3**).

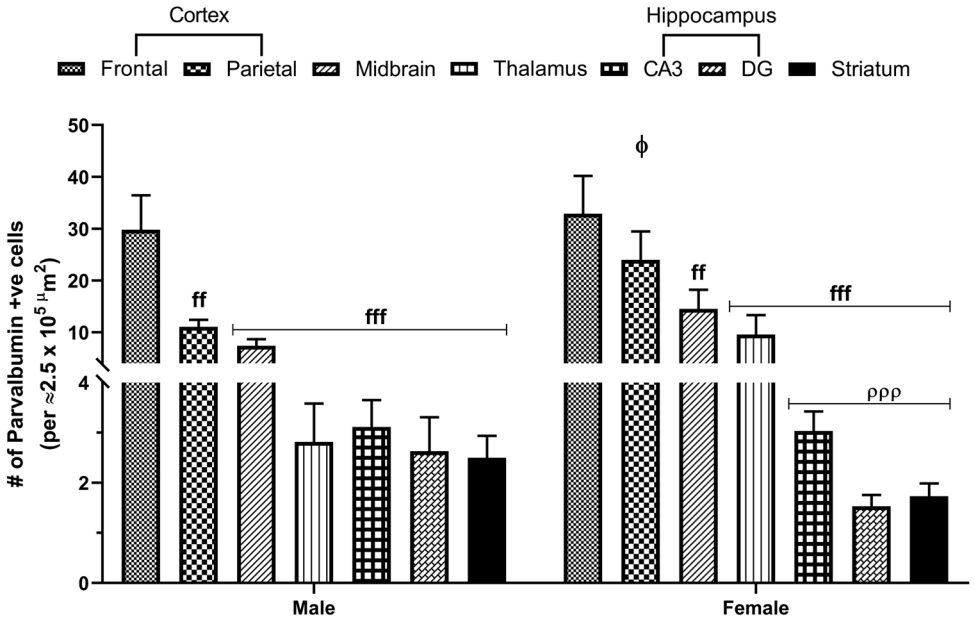

Fig. 3. Image J cell count of parvalbumin positive cells in the selected regions of the brain. Data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc tests. Significant difference between sex was further confirmed by t-test. $^{ff}p < 0.01$, $^{fff}p < 0.001$ compared to frontal cortex; $^{\rho\rho\rho}p < 0.001$ compared to parietal cortex; $^{f}p < 0.01$ between male and female for same region.

The ImmunoRatio plugin on Image J software was used to quantify immunoreactivity of parvalbumin expression. Two-ANOVA analysis of data obtained revealed no significant interaction ($p = 0.4938$; $F_{(6,69)} = 0.91$) between sex and brain regions, as well as no sex effect ($p = 0.5373$; $F_{(1,69)} = 0.38$). However, analysis revealed significant region specific effect ($p < 0.001$; $F_{(6,69)} = 64.22$). Bonferroni's post-hoc test revealed significantly lower ($p < 0.001$) parvalbumin expression in striatum compared to the other regions examined in male rats. Additionally, both regions of hippocampus examined, the CA3 and DG, showed significantly lower ($p < 0.01$) expression compared to thalamus, while only the DG showed significant lower ($p < 0.05$) expression compared to the frontal cortex. In the female rats, the parvalbumin expression in the striatum is also significantly lower ($p < 0.001$) compared to other regions. No other significant difference is seen. Similarly, no significant differences between the sexes are revealed (**Fig. 4**).

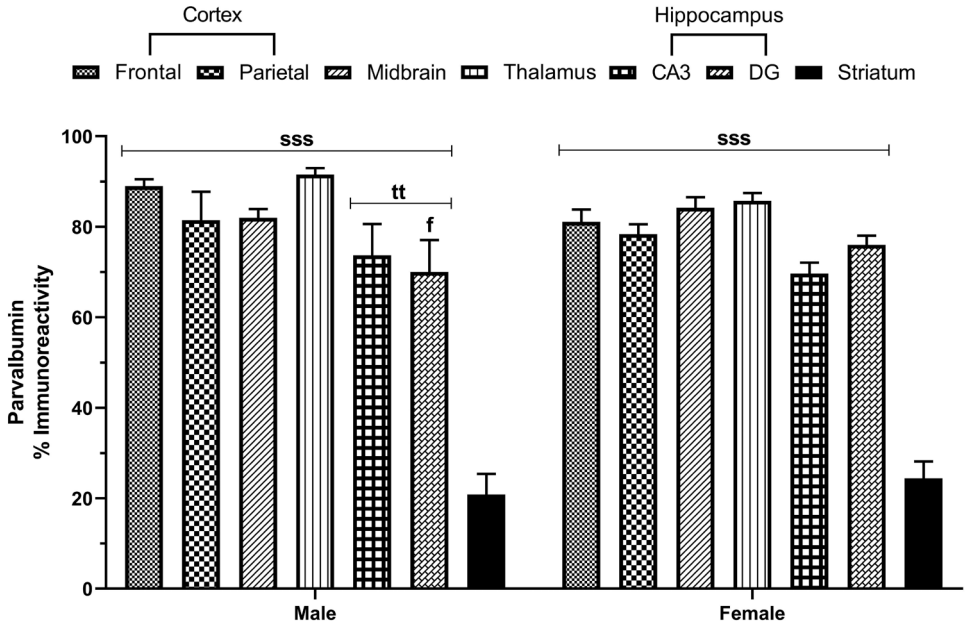

Fig. 4. ImmunoRatio analysis of parvalbumin immunoreactivity in the selected regions of the brain. Data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc tests. $^{f}p < 0.01$ compared to frontal cortex; $^{tt}p < 0.01$ compared to thalamus; $^{sss}p < 0.001$ compared to striatum.

Discussion

The current study evaluated parvalbumin distribution across several brain regions (frontal and parietal cortex, midbrain, thalamus, hippocampal CA3 and DG, and striatum), as well as examined the sexual dimorphism on the neuroanatomical distribution pattern of parvalbumin-expressing cells in the cerebrum.

The study demonstrates the differential distribution of parvalbumin-expressing cells across the above brain regions. Previous reports provide evidence that neuron and neurotransmitter distribution and axonal connection vary substantially across different cortical and subcortical areas [1,27]. In line with this, the findings here showed that parvalbumin-expressing neurons are more abundant in the frontal cortex, an area known for its immense importance in sensorimotor functions. Unsurprisingly, Kim and colleagues [15] highlighted the importance of parvalbumin neurons in sensory-motor cortical areas. They also noted that parvalbumin neurons appear to be dominant in these areas, which is similar to an earlier observation by an earlier study [14].

Interestingly, our results show a similar level of parvalbumin immunoreactivity in the subcortical regions (except for the striatum) compared to the cerebral cortex, despite the lower number of parvalbumin-expressing cells in the subcortical regions. Particularly, the thalamus, which showed a less parvalbumin-expressing cells compared to the cortex in both males and females, had higher parvalbumin immunoreactivity

compared to other groups, including the cortex, albeit not significant. This is indicative of strong parvalbumin-neuronal connections in these regions. For instance, although parvalbumin cells make up a small percentage of neurons in the hippocampus, they have the strongest inhibitory effect on the population of projection neurons [20], by forming a vast number of synapses amongst themselves and other neurons [8]. Likewise, these parvalbumin-expressing cells are characterized by their ability to form long-distance projections [28]. Therefore, it is possible that the regions with fewer soma of parvalbumin-expressing neurons might be receiving several axonal projections from other regions and exhibiting numerous synaptic connections.

Finally, the study examined the effect of sex dimorphism on parvalbumin distribution in the cerebrum. This is imperative to better understand the sex-related differences in the progression of some neurologic disorders. For instance, an earlier report showed a larger reduction of hippocampal parvalbumin-positive neurons in male schizophrenic patients compared to their female counterparts [30]. Further, a study in juvenile rats has shown that males generally had higher parvalbumin-positive interneurons in the upper part of the frontal cortex, but lower numbers in the dorsal hippocampus, compared to age-matched females [25]. Other preclinical studies indicate decreased parvalbumin-positive cells in males after prenatal stress, whereas females show more deficits after neonatal and adult stress [26]. In the present study, only in the parietal cortex did we observe a significant difference in parvalbumin distribution between the sexes, with females exhibiting a significantly higher number of parvalbumin-positive cells compared to males. Overall, these indicate sex-specific differences in parvalbumin distribution that could impact its role in the pathogenesis of brain disorders. Perturbations to parvalbumin interneurons have been linked to the pathophysiology of various neuropsychiatric, neurodevelopmental and/or neurodegenerative disorders, including autism, schizophrenia, bipolar disorders, Alzheimer's disease, among others [23]. Hence, understanding sex differences in parvalbumin distribution holds translational value to unravel sex-linked phenomena in the impact, distribution and severity of these brain disorders.

Conclusions

The study demonstrates that parvalbumin-expressing cells distribution is widespread across the cerebrum, with a higher population in the cortex compared to subcortical regions. However, levels of expression are similar across most major brain regions (except for the striatum), suggesting extensive connections of parvalbumin neurons. Also, the study indicates potentially sex differences in parvalbumin distribution in the cerebrum.

Limitations of the study: The study, however, highlights some limitations. Firstly, though the study sample size is based on PS Power analysis that indicates n = 6 is adequate, we do acknowledge that inter-individual variability could impact the findings. Further, immunostaining used here reveals broad types of parvalbumin-positive cells and could not account for the identification of subtypes. Also, we have not used sections from the whole brain, but serial sections were obtained from 2-3 mm lateral to midline as already described in methods, hence certain cerebral subregions might not have been fully represented. Finally, the use of immunohistochemistry may

produce results slightly different to other methods of cell visualization such as utilizing transgenic models.

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