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Cerebellar immunoexpression patterns of calcium-binding proteins in rats: potential sex-specific differences

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Abstract

Calcium ion (Ca^{2+}) is involved in various neurochemical signalling that contributes to neuronal activation, neurotransmitter modulation and synaptic connections. Hence, it is homeostatic regulation in the brain is of vital importance. Calcium-binding proteins (CaBPs) are key regulators of intracellular Ca2+ in the brain. The major CaBPs are calbindin, calretinin, and parvalbumin and are also markers for variety of neuronal subtypes in the brain. Understanding the distribution of brain neurons positively expressing these CaBPs as well as potential sex dimorphism in their expression is of huge relevance given their role in brain disorders. The present study examines calbindin, calretinin, and parvalbumin immunoreactivity in the cerebellum and any potential sex-specific differences. The results showed marked expression of these CaBPs in the cerebellum with calbindin and parvalbumin dominantly reactive in the molecular and Purkinje layers, and calretinin most predominant in the granular layer. Also, we see significantly higher calbindin immunoreactivity in male rats compared to female. While calretinin and parvalbumin immunoreactivity were also higher in males, this effect however did not reach significant levels. In conclusion, the present study demonstrates differential distribution patterns of the major CaBPs, calbindin, calretinin and parvalbumin, in the cerebellum and possible sex-specific differences in their immunoexpression.

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Introduction

Calcium in its ionic form, Ca^{2+}, plays many modulatory in the human body, particularly in the central nervous system (CNS), where it influences numerous neurochemical activities including neuronal activation, synaptic communication, and neurotransmitter modulation. Therefore, a homeostatic balance of intracellular Ca^{2+} levels is vital within the CNS, and disturbances have been linked to several neurological disorders including neurodegenerative diseases such as Alzheimer's and neuropsychiatric and neurodevelopmental disorders such as schizophrenia, autism amongst others (Fairless *et al.*, 2019; Ijomone *et al.*, 2019). Predominant among the several regulators of Ca^{2+}, are protein molecules called calcium-binding proteins (CaBPs). Many CaBPs exist within a large family called EF-hand CaBPs, which play significant roles in intracellular Ca2+ homeostatic regulation by binding and transportation of the ion. The major EF-hand CaBPs are calbindin D28-k (simply called calbindin), calretinin, and parvalbumin. Each of these proteins possess high-affinity for cytosolic Ca2+ thus involved in regulation of free Ca2+ in neuronal cytosol. Importantly, these proteins have been demonstrated as important neuronal marker in the brain, particularly for distinct subgroups of GABAergic interneurons throughout the brain (Ahn *et al.*, 2017; Berg *et al.*, 2018; Ijomone *et al.*, 2019).

The cerebellum is the small ovoid-shaped region at the back of the brain of many vertebrates. It is gross anatomically distinct from the brain other parts and is mostly involved in modifying signals from higher brain centres. The cerebellum is well-known for coordinating motor activities, but is now strongly linked to other higher order roles including cognitive, emotions and language. The cerebellum is home to a large number neuron with many of such been them expressing the EF-hand CaBPs calbindin, calretinin, and parvalbumin (Brandenburg *et al.*, 2021; Miguel *et al.*, 2021). Furthermore, cerebellar dysfunction is associated to a variety of neurological diseases including motor and non-motor conditions such as Parkinson's, Huntington's, Multiple Sclerosis, Autism, Schizophrenia, amongst others (Reeber *et al.*, 2013). Given the role of the CaBPs several neural signalling activities and the importance of the cerebellum in many brain disorders, understanding their distribution pattern of neurons expressing these CaBPs in the cerebellum is a particularly useful resource in neurosciences. Also, very important will be understanding potential sex dimorphism in expression patterns of these CaBPs. Hence, our present study examined calbindin, calretinin, and parvalbumin expression patterns in the cerebellum via immunolabeling, as well as assessed potential sex-specific differences in their expression patterns.

Methods

Animals

Twelve adult rats (Sprague-Dawley strain) aged 10 weeks were procured and used for this study. Rats were of both sexes (6 males and 6 females) with average weight of 339±4.84 g for males, and 215±4.59 g for females. All protocols were performed in adherence to the guidelines for the care and use of laboratory animals (NIH, 2011). Animals were housed in standard plastic cages with free access to food and water, and kept under 12-hour light-dark cycle.

Rats were allowed to acclimatize for 3 weeks post-procurement, and then 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. The cerebellar region was targeted via mid-sagittal sections. Thereafter, we performed immunohistochemistry procedure as previously established (Ijomone *et al.*, 2018). Sections were dewaxed and taken to water. Antigen retrieval was performed via citrate-based heat mediated protocol. Endogenous peroxidase blocking was performed in hydrogen peroxide solution (0.3% in Tris Buffered Saline) for 10 mins. Sections were treated with 2.5% normal horse serum (Vector® #MP-7401) for 30 mins, prior to primary antibody incubation in anti-

calbindin (Abcam, USA; ab49899) at 1:200 dilution, anti-calretinin (Abcam, USA; ab203055) at 1:200, anti-parvalbumin (Novus Biologicals, USA; NB120-11427) at 1:1000 dilution, for 1 h at room temperature. Sections were treated in secondary ImmPRESS™ HRP Polymer Anti-Rabbit IgG (Vector® #MP-7401), and brown immunoreaction developed with DAB Peroxidase Substrate Kit (Vector® #SK-4100). Sections were then counter-stained in haematoxylin, and cover-slipped using permount reagent.

Imaging and Quantification

Brain digital imaging was performed using the Pannoramic 250 Flash II slide scanner (3D Histech, Budapest, Hungary) at 20x objective. Ten random non-overlapping images of the cerebellar region were obtained at x200 magnification (area size = ≈2.5 x 10^5 μm^2) from each animal using the CaseViewer Imaging software (3D Histech, Budapest, Hungary). Images were then imported onto the NIH, USA open-source Image Processing and Image Analysis in Java (ImageJ) program for quantification of immunoreactivity using the ImmunoRatio plugin which separates and quantifies the percentage of DAB (positive immunoreactivity) by digital colour deconvolution (Erukainure *et al.*, 2019).

Statistics

Data are presented as mean ± SEM. Data were analysed on GraphPad Prism 8 software (GraphPad Inc, USA) by student t-test to check statistical differences between the sexes. Values of $p < 0.05$ were considered statistically significant.

Results

Imaging of immunostained sections of the cerebellum showed marked expression of calbindin (Fig. 1A), calretinin (Fig. 2A) and parvalbumin (Fig. 3A) in both male and female rats.

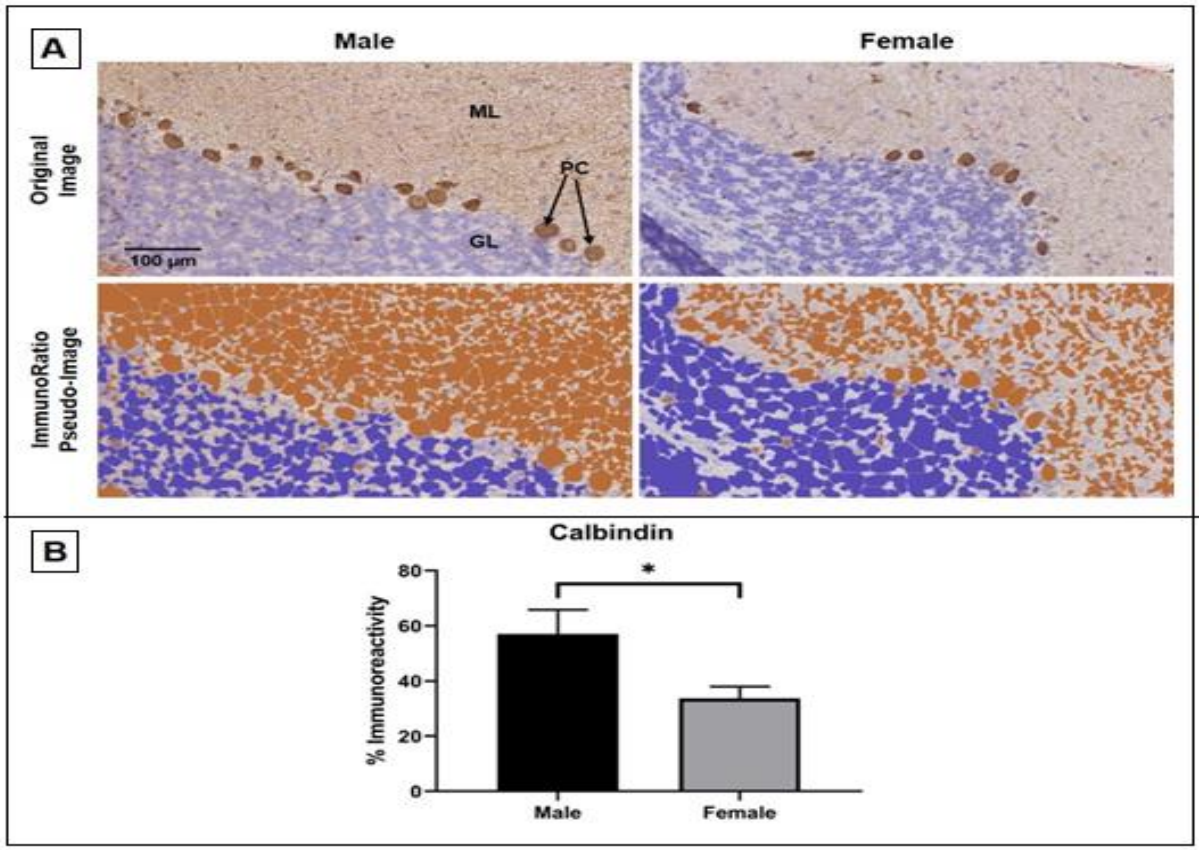

Fig. 1. A: Calbindin expression in cerebellum of male and female rats. Original images are accompanied by Pseudo-Image generated with ImmunoRatio plugin in Image J software. ML – molecular layer; PC – Purkinje cells/neurons; GL – granular layer. B: ImmunoRatio analysis of calbindin immunoreactivity. *$p < 0.05$ between male and female rats using student t-test.

We observe strong immunopositivity in the large-sized Purkinje neurons of the cerebellum for the three CaBPs evaluated. Furthermore, the molecular layer of the cerebellum shows marked immunoreactivity for calbindin and parvalbumin but not calretinin. Many of small-sized cells in the molecular layer which include stellate and basket cells show strong calbindin and parvalbumin immunopositivity including their axons. On the other hand, the granular layer of the cerebellum shows marked immunoreactivity for calretinin but not calbindin and limited immunoreactivity to parvalbumin.

ImageJ quantification showed that significantly ($p < 0.05$) higher calbindin immunoreactivity in males compared to females (Fig. 1B). No such significant different between the sexes is observed for calretinin (Fig. 2B) and parvalbumin (Fig. 3B) expression, although expression were higher in males than females.

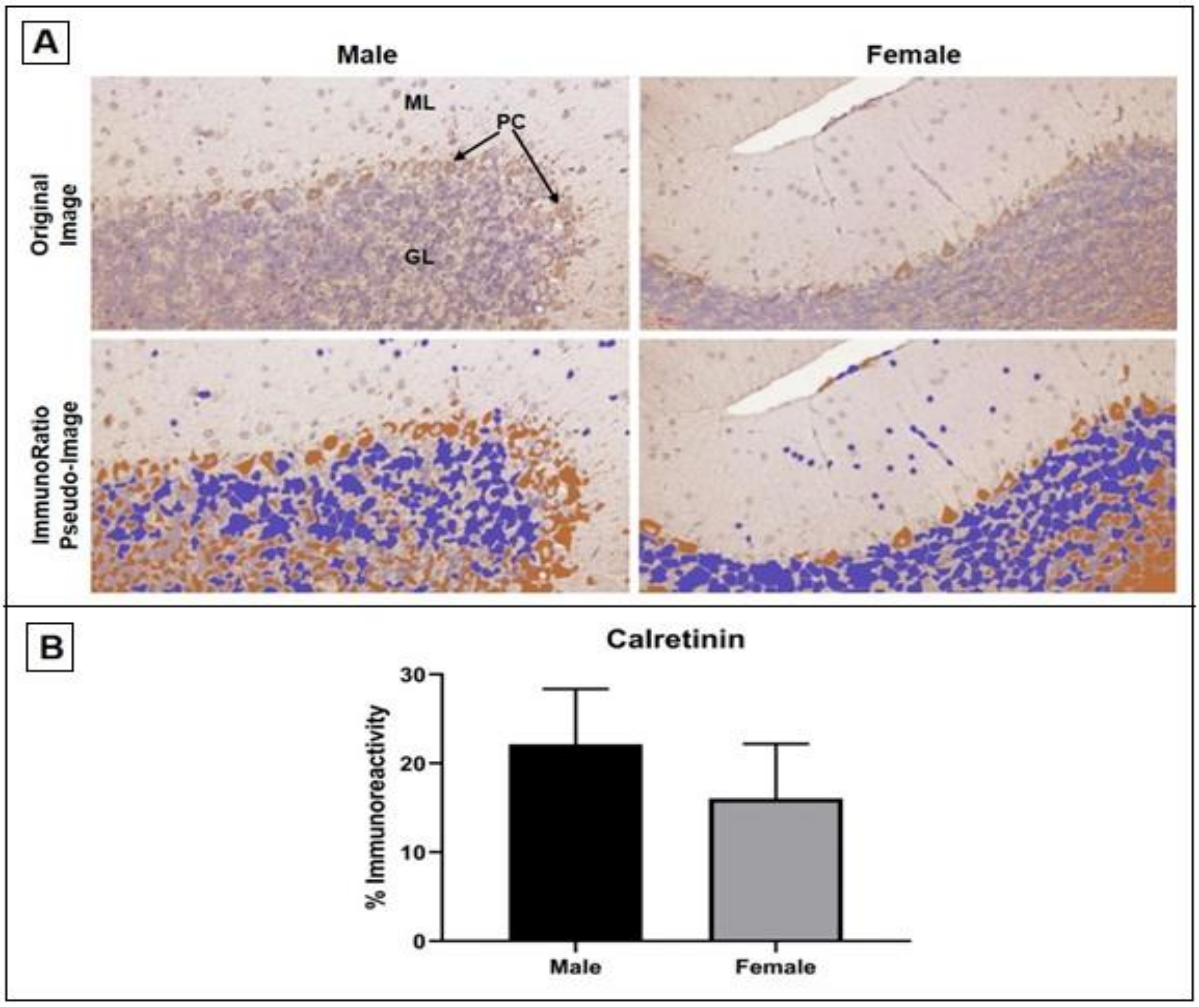

Fig. 2. A: Calretinin expression in cerebellum of male and female rats. Original images are accompanied by Pseudo-Image generated with ImmunoRatio plugin in Image J software. ML – molecular layer; PC – Purkinje cells/neurons; GL – granular layer. B: ImmunoRatio analysis of calretinin immunoreactivity.

Discussion

The present study evaluated immunoexpression patters of CaBPs, calbindin, calretinin and parvalbumin in the cerebellum of rats, as well as examined sex specific differences in the immunoreactivity of these CaBPs. Our show strong positive immunoreactivity for all CaBPs examined in the cerebellum. This supports several previous studies which have shown that neuronal subtypes in the cerebellum including the small-sized stellate and basket cells of the molecular layer, large-sized Purkinje cells in the single-layer intermediate space,

and small-sized granule cells in the granular, are immunopositive for CaBPs (Bastianelli, 2003; Flace *et al.*, 2014; Brandenburg *et al.*, 2021).

Results from the current study show that calbindin and parvalbumin are most strongly immunoreactive in the cells of the molecular and Purkinje layer, and to lesser extent in the granular layer. Contrastingly, calretinin immunoreactivity is dominant in the granular layer of the cerebellum. Previously, other authors have also noted that Purkinje cells of the cerebellum contain CaBPs calbindin and parvalbumin, whereas calretinin is exclusive to the cells in cerebellar granular layer (Fairless *et al.*, 2019; Miguel *et al.*, 2021). Our results partly support this, as we have also observed obvious calretinin immunoreactivity in the Purkinje cells, albeit to a lesser extent compared to calbindin and parvalbumin. However, expression of CaBPs, calbindin and parvalbumin, observed in the current studies are consistent with earlier reports which have shown strong immunoreactivity in the molecular layer but weak or absence of immunoreactivity in the granular layer (Flace *et al.*, 2014; Brandenburg *et al.*, 2021). On its part, calretinin immunoreactivity in the cerebellar molecular layer is almost non-existent.

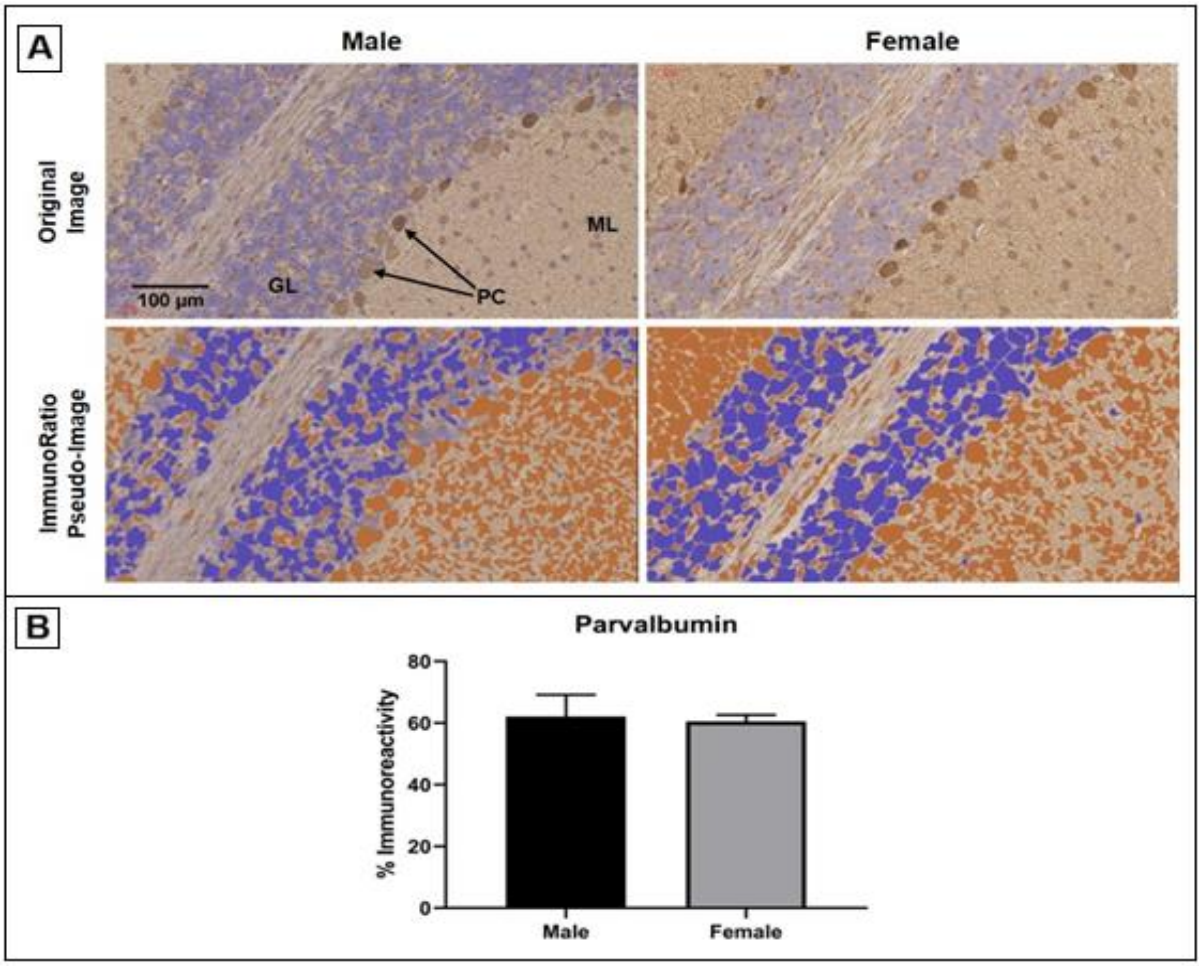

Fig. 3. A: Parvalbumin expression in cerebellum of male and female rats. Original images are accompanied by Pseudo-Image generated with ImmunoRatio plugin in Image J software. ML – molecular layer; PC – Purkinje cells/neurons; GL – granular layer. B: ImmunoRatio analysis of parvalbumin immunoreactivity.

Furthermore, the study examined sex-specific differences in cerebellar immunoreactivity of the CaBPs. The result showed significantly higher cerebellar calbindin immunoreactivity in males compared to females, but no such significant difference in cerebellar calretinin and parvalbumin immunoreactivity, albeit higher in males. Previous studies examining subcortical structures in the higher

brain centres have reported higher calbindin and calretinin expression in males compared to females. Brager and colleagues previously reported higher immunoexpression of calbindin and calretinin in the hypothalamus of juvenile male rats compared to their female littermates (Brager *et al.*, 2000). Other authors also showed higher cell number and volume of calbindin positive neurons in the medial preoptic nucleus and the bed nucleus of the stria terminalis of male adult mice compared to their female counterparts (Moe *et al.*, 2016). Furthermore, one study showed differential sex-specific difference in parvalbumin expression across several brain regions. The study showed higher parvalbumin positive neurons the striatum of female rats compared to males, but an opposing effect with males having higher parvalbumin neurons in the cortical amygdala and nucleus accumbens. The same study also observed no such significant sex difference in the hippocampus and medial amygdala nucleus (Stefanova *et al.*, 1997). Similarly, a more recent report showed no significant sex difference in the hippocampus and surrounding neocortex (Griffiths *et al.*, 2019). Contrastingly, other authors have reported greater density of parvalbumin positive neurons in males compared to females (Tucker *et al.*, 2019). Taken together, the present study suggests that the CaBPs expressions in the cerebellum may also be sexually dimorphic as observed in other brain regions. Providing more information of potential sex-specific difference in CaBPs positive neurons may prove useful in unravelling impact of sex on progression of some neurological disorders, particularly the several neuropsychiatric, neurodevelopmental and neurodegenerative disorders that have been linked to activity of these CaBPs.

Conclusion

In conclusion, the present study demonstrates differential distribution patterns of the major CaBPs, calbindin, calretinin and parvalbumin, in the cerebellum and possible sex-specific differences in their immunoexpression. It is noteworthy that the results obtained in present study are not without some limitations. Other approaches of cell visualization such utilizing transgenic models may produce slightly different results compared to immunohistochemistry method. Also, here we have used serial sections from mid-sagittal cuts through the cerebellum and not cuts through the whole cerebellar region.

Conflict of Interests: The author declares no conflict of interests.

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