Neurohistology: How to Identify Neuronal Proteins in Distinct Brain Regions and Cell Types

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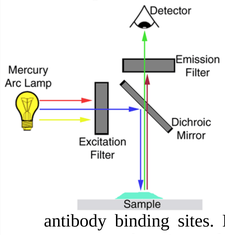
Abstract

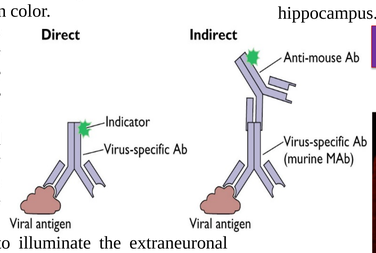
Parvalbumin is a calcium binding protein that has been intensively studied in cortical inhibitory interneurons. However, less clear is the notion whether parvalbumin expression is restricted, in general, to inhibitory neurons. In fact, recent work demonstrates that cortico-striatal neurons can co-express the excitatory transmitter, glutamate, along with parvalbumin. It is not known if this is exceptional or if many glutamergic populations also express parvalbumin. It has long been postulated that calcium binding proteins like parvalbumin play a prominent role in both short and long term synaptic plasticity. Calcium signaling is responsible for many physiological processes involving synaptic activity; in procedures like cell-cycle regulation, secondary messenger production, and muscle contraction that require Calcium signaling, the differential expression of Calcium buffers is thought to fine-tune the regulation of these processes. In this study, we evaluate evidence for parvalbumin expression in glutamergic populations of excitatory neurons in distinct brain regions of mice. Studies of differential parvalbumin neuronal expression may bring us a broader understanding of the physiological role of parvalbumin in synaptic transmission.

Background

To explore the differential expression of parvalbumin among cortico-striatal neurons and other glutamergic populations, or any specific region of the brain as a whole, it is imperative that a method is devised to make visible the anatomy of the tissue being studied and to discriminate with great specificity between areas of parvalbumin expression and those that lack expression. All this is accomplished through a combination of immunocytochemical, chemical, and transgenic staining. Each staining technique rests on the principle of fluorescence microscopy, and the use of molecules called fluorophores that selectively absorb and emit light at specific wavelengths. That way, when a strong light source illuminates the sample, only photons of certain wavelength are emitted back, allowing areas of interest marked by fluorophores to glow in a certain color.



Due to the acute specificity of antibodies binding to their target antigens, they prove an invaluable tool for immunohistochemistry staining. For our purposes, we used anti-parvalbumin as our primary antibody to selectively bind to parvalbumin. Our secondary antibodies conjugated with fluorophores then could selectively bind to the primary antibodies and illuminate the primary antibody binding sites. However, a counterstain is still needed to illuminate the extraneuronal anatomy; otherwise, only the parvalbumin regions would be visible and there wouldn't be any sense of directionality upon imaging. The NeuroTrace Blue fluorescent Nissl stain serves this purpose because it is selective for Nissl substance characteristic of neurons, providing a marker for the neuronal morphology and distribution. As for our manipulated variables in this study, we used transgenic mice expressing either vGAT-tdTomato (genetically engineered to fluoresce only inhibitory neurons) or vGLUT2-tdTomato (genetically engineered to fluoresce only excitatory neurons).

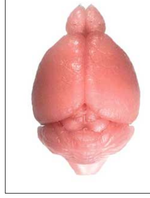


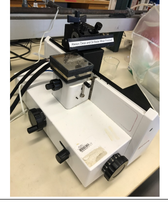
Methodology

Procedure

We used N=2 transgenic mice to identify excitatory (vGLUT2-tdTomato) or inhibitory neurons (vGAT-tdTomato). Parvalbumin-localization is assayed in transgenic brain sections by immunocytochemical staining with anti-Parvalbumin antibodies. Note: tdTomato red fluorescent protein has its peak excitatory spectra at 554 nm and emission spectra at 581 nm.

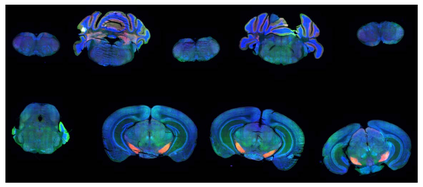
- After anesthesia overdose, mouse brains were fixed by transcardial perfusion and extracted from the skull. All animal procedures were approved by the UCSD IACUC.
- Brains were post fixed and then equilibrated in sucrose for sectioning at 40 µm on a freezing sliding microtome.
- Sections were collected in multiwell tissue culture dishes, washed with PBS buffer solution, and immunostained with anti-parvalbumin antibody and anti-mouse antibody conjugated with fluorophore(488 nm/510 nm)
- Control sections were run in blocking buffer and secondary
- After several rounds of washing with PBS, sections were then counterstained with NeuroTrace (435 nm/455 nm)
- Stained sections were mounted on glass slides, coverslipped and imaged at 435, 488, and 554nm



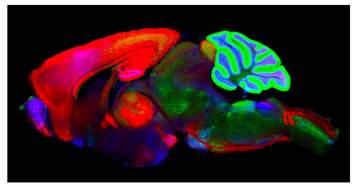


Scanned 3-Channel Fluorescencent Sections

vGAT-tdTomato Stained Tissue Imaged at 435 nm, 488 nm, and 554 nm



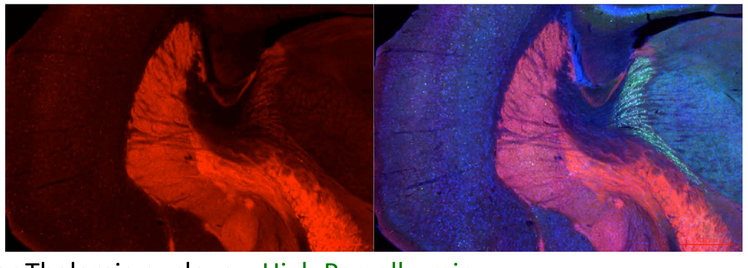
vGLUT2-tdTomato Stained Tissue Imaged at 435 nm, 488 nm, and 554 nm



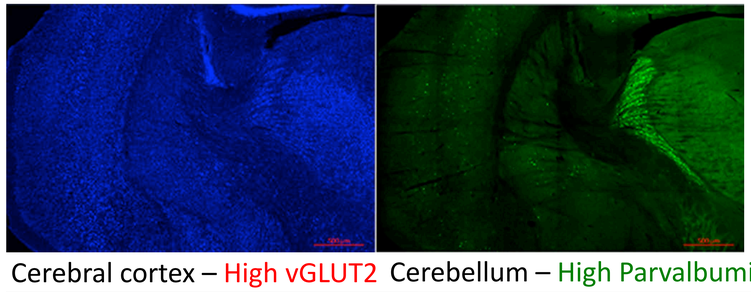
After various tissue sections were coverslipped and frozen overnight, fluorescence microscopy was performed in a huge Zeiss slidescanner at 435 nm, 488 nm, and 554 nm for the peak excitation levels of the blue Neurotrace, eGFP conjugated antibodies, and tdTomato fluorescent protein. The results of the imaging can be seen above, with the green hues generally indicative of relative of areas with high neuronal parvalbumin expression, and the red hues indicative of areas with high concentration of vGAT expressed transgenetically with the tdTomato. Areas most notably having high parvalbumin expression are the reticular thalamus, expressed by chandelier and basket cells in the cortex. In the cerebellum, parvalbumin is mostly expressed in molecular layer interneurons and Purkinje cells. vGAT expression occurs predominantly in the striatum and olfactory bulb, with fiber tracks extending down to the reticular substantia nigra in the midbrain sections, as well as the molecular layer of Lobules III-V and cerebellar nuclei of the cerebellum sections. On the other hand, areas having high vGLUT2 expression appear mostly in the cerebrum and cerebral cortex, more specifically the somatosensory and motor areas, visual cortex, and hippocampus. Sections stained with GLUT2 were sectioned sagitally for more emphasis on cerebral anatomy.

Results

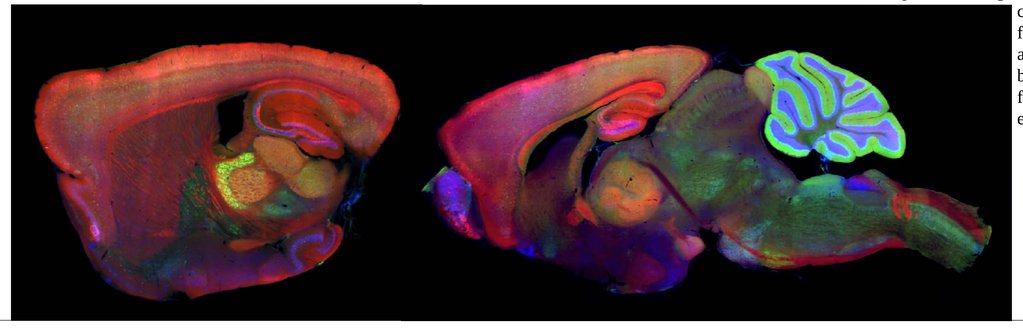
Striatum – High vGAT



Reticular Thalamic nucleus – High Parvalbumin

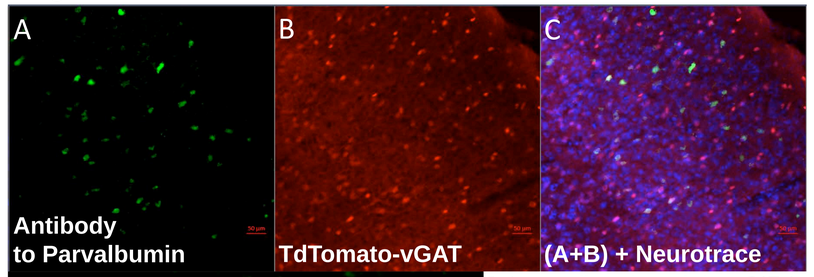


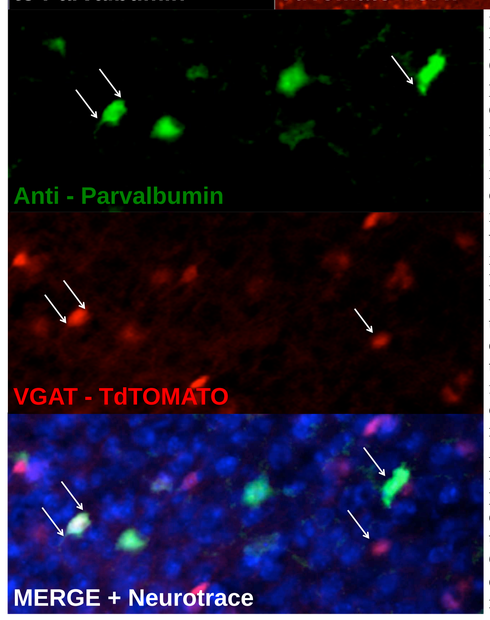
Cerebral cortex – High vGLUT2 Cerebellum – High Parvalbumin



Analysis of Coexpression in vGAT Cortical Tissue

Single section of cortex with three fluorescent labels





It is generally understood that vGAT is highly concentrated in the nerve endings of GABAergic neurons in the brain, whose principal role is reducing neuronal excitability with the inhibitory neurotransmitter GABA. For this reason, transgenic labeling of vGAT with tdTomato is particularly useful in accurate marking of only inhibitory neurons. From the imaging, it is clearly evident that there are instances when parvalbumin is not chiefly expressed in areas where vGAT is expressed. However, there are certain cortical areas where co-expression of both the vGAT and the parvalbumin occurs, manifested by the dual coloration of tdTomato and eGFP in the same area. On the zoomed images of figures A, B, and C, the white arrows correspond to individual neurons present in multiple channels upon imaging. Accordingly, it is possible for individual neurons to exhibit both pervalbumin proteins and vGAT. This evidence of co-expression is significant because it is proof that parvalbumin exhibiting neurons and GABAergic neurons are not mutually exclusive, and suggests that the mechanisms for vGAT and parvalbumin expression may be related in some way.

Discussion and Conclusions

From the results of this study, it is clear that parvalbumin expression is not restricted to inhibitory neurons. The explicit differences in areas stained by anti-parvalbumin and vGAT-TdTomato warrant the possibility that the mechanisms responsible for parvalbumin expression and vGAT (inhibitory) expression may be entirely separate, invalidating the previous notion that differential parvalbumin expression could serve as a good model for labeling inhibitory interneurons. Our findings are consistent with evidence that Parvalbumin is differentially expressed by inhibitory and excitatory neurons in different brain regions. To confirm these results, our stained sections can be imaged on a confocal microscope to verify co-expression. Interestingly, there were still instances throughout the study where parvalbumin and vGAT were co-expressed among specific neuronal populations, indicating that parvalbumin exhibiting neurons and GABAergic neurons are not mutually exclusive. Therefore, the underlying mechanisms driving parvalbumin and vGAT expression may still be interrelated, despite the divergent nature of the underlying mechanisms responsible for their expression. Given more time to continue my experimental research, I would investigate the mechanism actually responsible for parvalbumin expression, and more specifically, why parvalbumin is co-expressed among both cortico-striatal neurons, glutamergic neuronal populations, and areas of the brain lacking vGAT expression like the reticular thalamus. My possible experiments in the future would assess the expression of RNA for calcium binding proteins in inhibitory and excitatory neurons.

References

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Gene Expression Deficits in a Subclass of GABA Neurons in the Prefrontal Cortex of Subjects with Schizophrenia
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