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Establishing an immunohistochemical protocol for visualization and quantification of noradrenergic fibers and localization of α1, α2, and β1 receptors in the hippocampus of rhesus macaques

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INTRODUCTION

The locus coeruleus (LC) is a brainstem nucleus known best for being the primary site of noradrenaline (NA) production and has wide projections throughout the cortex. NA acts via 3 classes of receptors (α1, α2, β) and this signaling is critical for optimization of cognitive performance.

NA fiber density has not been investigated with density of all 3 NA receptor types, and no protocol exists for fluorescence immunostaining analysis of NA receptors in primates. Here we developed a protocol for staining the NA system along with other cell types known to express noradrenergic receptors.

METHODS

Immunocytochemistry Procedure

Tissue preparation: Tissue from a cohort of adult and aged rhesus macaques was used for this study. Following perfusion, brains were fixed in a solution of 4% PFA and 30% sucrose at 4°C. All brains were cut into 30µm sections in the coronal plane.

Immunostaining Procedures: On the day of staining, sections were first washed in Tris Buffer Solution (TBS). Tissue then underwent an antigen retrieval procedure. Before immunostaining procedures, tissue was blocked in a solution containing TBS, 3% Normal Donkey Serum, and 0.3% TritonX-100. Brain sections were incubated in primary antibodies (see Table 1) overnight. The next day, the tissue was washed in TBS, incubated in secondary antibodies for 2 hours, washed again in TBS then mounted with 80% glycerol in TBS.

Control Immunostaining Experiments: Each antibody was tested at multiple concentrations to find the optimal combination of staining. Control experiments were performed to prove specificity of primary and secondary antibodies. Primary antibodies were preadsorped with a peptide corresponding to their immunogen sequence and then incubated with the tissue as described above (Figure 1). Anti-adrenergic receptor antibodies were incubated with all other primaries and secondaries, and this was compared to preparations with all primaries but only the secondary for the receptor (Figure 2). In preparations where primary antibodies were omitted, secondary antibodies did not show any detectable staining.

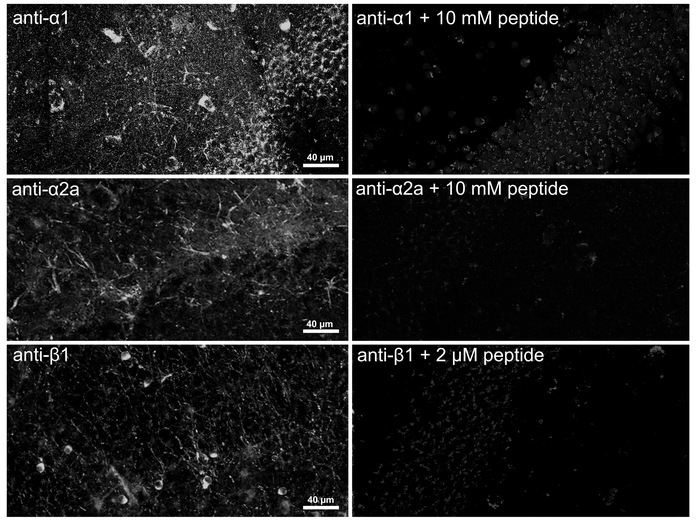
Visualization: Tissue was imaged at 10, 20, and 40x using a Zeiss LSM 880 confocal microscope. Zen Blue was used to process images by separating individual channels and removing autoflourescing elements such as lipofuscin through a process known as linear unmixing*.

Table 1: Antibodies and markers used

Primary Antibodies	Secondary Antibodies
Noradrenergic fibers: Sheep anti-Dopamine ß Hydroxylase or DBH (1:1000, MilliporeSigma AB1537)	Alexa Fluor488 AffiniPure F(ab')2 Fragment Donkey Anti-Sheep (1:500, JacksonImmunos)
Rabbit Anti-a1 Adrenergic Receptor antibody (1:125, MilliporeSigma A270)	Alexa Fluor647 AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit (1:200, JacksonImmunos)
Rabbit Anti-ß1 Adrenergic Receptor antibody (1:100, Novus NBP2-39091)	Cy3 Donkey anti-Guinea Pig (1:1000, VWR International)
Rabbit Anti-alpha 2a Adrenergic Receptor antibody (1:200, Abcam Ab85570)	Cy3-conjugated Streptavidin (1:1000, VWR International)
Microglia: Guinea Pig anti-IBA1 (1:1000, Synaptic Systems 234308)	
Astrocytes:Guinea Pig anti-Glial Fibrillary Acidic Protein or GFAP (1:1000, Synaptic Systems 173004)	
Vasculature: Solanum Tuberosum Lectin (STL) –Streptavidin (1:200, VWR International B-1165-2)	

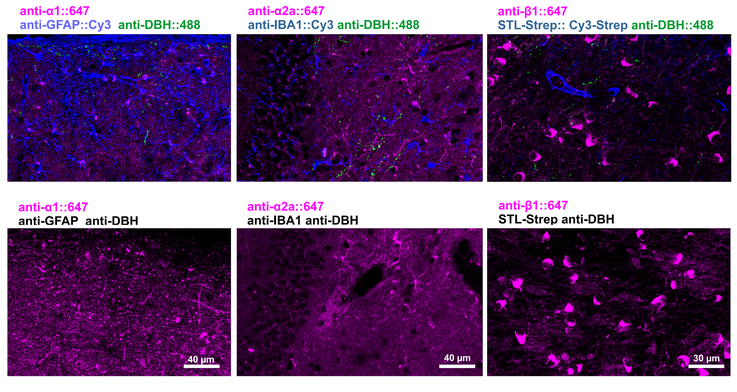
RESULTS

Figure 1: Characterization of the immunostaining specificity of anti-α1, anti-α2a, and anti-β1 receptor antibodies



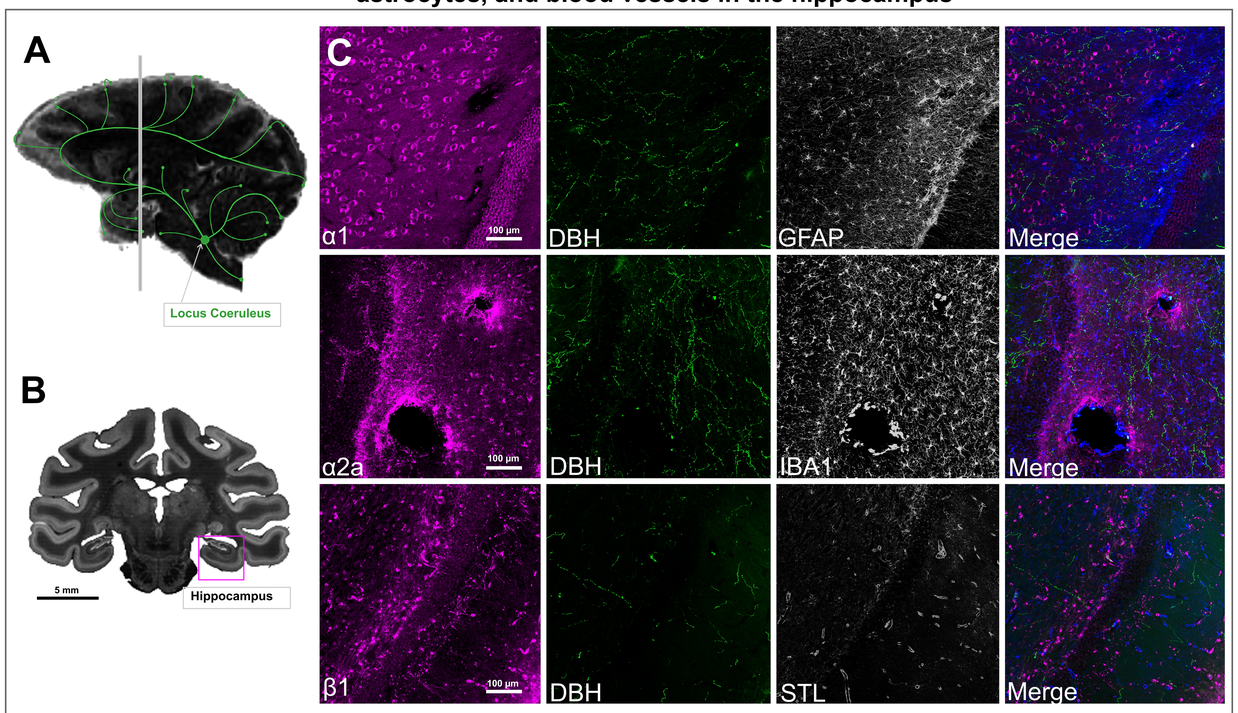
To prove specificity of the tested adrenergic receptor antibodies, each antibody was pre-incubated with a corresponding peptide that had its specific antigen sequence. Confocal images of the preadsorped (right panel) and control stains (left panel) indicated that the anti-α1 (top panel), anti-α2a (middle) and anti-β1 (bottom) antibodies are specifically labeling their corresponding receptors in this tissue, as preadsorption of antibodies with the peptide abolished specific staining.

Figure 2: Test of primary antibody layer interactions in triple immunofluorescence staining



Primary antibody layers did not interact with each other in simultaneous immunofluorescence triple staining in our chosen concentrations.

Figure 3: Visualization of the adrenergic receptors alongside adrenergic fibers, microglia, astrocytes, and blood vessels in the hippocampus



Tissue sections corresponding to the rhesus macaque Paxinos atlas 80 were selected for staining for the simultaneous visualization of noradrenergic fibers and receptors. **A)** A schematic distribution of noradrenergic projections overlayed onto an MRI scan of an animal used for staining. A gray bar denotes the approximate area from which the sections were selected in **B**. **B)** A Nissl-stained section adjacent to one of the sections used for staining in **C**. **C)** Simultaneous triple staining of each corresponding adrenergic receptor with noradrenergic fibers (anti-DBH), microglia (anti-IBA1), astrocytes (anti-GFAP), and vascular (STL) markers.

CONCLUSIONS

We have developed a staining protocol for the visualization of noradrenergic fibers and receptors in the rhesus macaque hippocampus. Next, we developed a triple immunofluorescence staining with astrocytes, microglia, and blood vessels for visualization of noradrenergic receptors on different cell types.

Staining of the α1 receptor was visible on large cell bodies as well as fibers. α2a was predominantly found on fibers, but was present on some cell bodies. α2a also colocalized with microglia as well as vasculature. β1 staining was also evident on both cell bodies and fibers, and colocalized with vasculature. Notably, cells immunopositive for the β1 receptor are smaller than those marked by the α receptor antibodies.

A future direction of this project is to analyze age-related alterations in the density of noradrenergic receptors and fibers.

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