

Conserved molecular and cellular features of the Islands of Calleja

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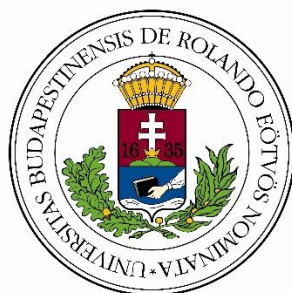
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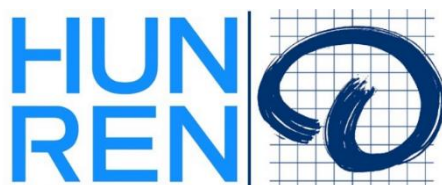
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Summary

The Islands of Calleja (IoC) is an understudied brain structure located in the basal forebrain. Although recent rodent studies suggest a potential role for the IoC in affective disorders, it is unclear whether the presence or organization of this structure is evolutionarily conserved across species. Our research group and other previous studies revealed that the IoC "islands" are formed by densely packed small granule cells (GCs), but their exact cellular architecture remains elusive. Our precise aim was to investigate the molecular and cellular features of the IoC across species. By performing a comprehensive fluorescent labeling analysis, I found that the IoC is an evolutionarily conserved anatomical structure, present in brain sections of mice, dogs, monkeys, and humans. I discovered that the cellular composition of the IoC is more complex than previously thought, with various cell types embedded within the GC clusters. By using key neurochemical markers for striatal medium spiny neurons (MSNs) and interneurons, I identified multiple MSN- and interneuron-like cells in the mouse IoC, and their presence in the islands is an evolutionarily conserved feature. These findings provide a foundational framework for future IoC studies and may help to uncover the exact pathophysiological role of this enigmatic brain region.

List of abbreviations

ChAT – Choline acetyl-transferase

CR – Calretinin

DAPI – 4',6-diamidino-2-phenylindole

DARPP-32 – Dopamine- and cAMP-regulated neuronal phosphoprotein

Fluo-CAR – Fluorescent Cariprazine

GFAP – Glial fibrillary acidic protein

GCs – Granule cells

IBA1 – Ionized calcium-binding adapter molecule 1

IN – Interneuron

IoC – Islands of Calleja

MSNs – Medium spiny neurons

NAcc – Nucleus Accumbens

nNOS – Neuronal nitric oxide synthase

PV – Parvalbumin

Introduction

Globally, affective disorders impact one in four people, yet effective treatment options for these conditions remain very limited. Cariprazine, a third-generation antipsychotic has shown high efficacy in treating bipolar depression, and has a unique potency to ameliorate affective symptoms associated with schizophrenia^{1, 2, 3}. Despite its clinical success, its precise mechanism of action remained elusive. Recently, our group has developed a technique to visualize drug binding sites in brain tissue in a cell-type specific manner, allowing us to investigate the precise distribution of Cariprazine⁴. With the help of a fluorescent analogue, we discovered that fluorescent Cariprazine (Fluo-CAR) shows the highest intensity signal close to cell-dense clusters in the basal forebrain, the so-called Islands of Calleja (IoC). At the molecular level, we have discovered that the antipsychotic's key molecular targets are D₃ dopamine receptors on the axons of granule cells (GCs), which represent the principal cell-type within this region. Although the cell dense-islands and their Fluo-CAR rich hilus appear as separate clusters on coronal sections, our 3D reconstruction of high affinity binding sites revealed that Fluo-CAR binds to a surprisingly large continuous region at the ventromedial border of the ventral striatum, representing the IoC complex.

The ventral striatum is a well-known subcortical brain region, fundamental in reward processing and motivation. It has been identified as a key site for neuroplastic events underlying addictive behaviors⁵. Additionally, abnormalities in ventral striatal activity have been associated with mood disorders⁶.

Despite the strategic anatomical localization of the IoC, this region is not in the focus of contemporary neuroscience. Even though recent studies – using opto- and chemogenetic techniques – revealed, that the activity of IoC may modulate depressive like behavior in mice⁷, the precise physiological or pathophysiological role of GCs remains unknown. Furthermore, all previous findings have been derived from studies on the mouse brain, and very little is known about the IoC in other species, including its presence in humans. We aimed to bridge this critical gap by conducting a comparative anatomical study of the IoC. Our findings highlight the potential neuropsychiatric significance of this largely understudied brain region.

Objectives

While it has been linked many times to affective disorders, the few studies about the IoC are based mainly on findings on the mouse brain, with minimal data available about the IoC in other species, or its presence and precise anatomical location in humans. To address this significant gap, we conducted a comparative anatomical study of the IoC complex. Using immunohistochemistry, we sought to address two key questions to facilitate future translational research on this brain region.

- First, I aimed to investigate whether the unique morphology and position of the islands we found in the mouse brain are conserved across species.
- Secondly, I aimed to identify evolutionary conserved molecular and cellular features of the IoC, by performing a comprehensive fluorescent labelling analysis.

Materials and methods

All experiments were conducted using the resources of the Laboratory of Molecular Neurobiology, HUN-REN Institute of Experimental Medicine (IEM). Human samples were obtained from the Human Brain Research Laboratory (IEM), Monkey samples were obtained from the Thalamus Research Group (IEM), and dog samples were obtained from Enikő Kubinyi's Laboratory (HUN-REN ELTE).

Animals

All experiments were approved by the Hungarian Committee of the Scientific Ethics of Animal Research and were carried out according to the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, Section 243/1998), in accordance with the European Communities Council Directive of November 24, 1986 (86-609-EEC; Section 243/1998).

Mice were kept under approved, specific-pathogen-free laboratory conditions (12-h light/12-h dark cycle, 22–24°C, 40–70% humidity). All efforts were made to minimize pain and to reduce the number of animals used. C57BL/6 mice were obtained from Charles River Laboratories.

Perfusion & Sectioning

Mice were anesthetized with intraperitoneal injection of avertin (1.25% v/v, Sigma), then transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB; pH 7.4) and fixed overnight at 4°C. 40-µm-thick brain slices were sectioned with a Leica VT-1200S Vibratome after embedding the samples in agar.

Immunolabeling

To obtain high-quality, and easily analyzable microscopic images, I used immunohistochemical methods. Specific binding sites in the samples were blocked, and the tissues were loosened as a preliminary step. Mice samples were treated with 5% normal donkey serum (NDS), 1% bovine serum albumin (BSA), 0.3% Triton in TBS (Tris-buffered saline) for 45 min for blocking and permeabilization and labeled overnight with primary antibodies (details listed in the Primary antibodies table, T1). Following three 10 minute washing steps in TBS, the corresponding fluorescent secondary antibodies (details listed in

the Secondary antibodies table, T2) were incubated for 2 hours. To decrease autofluorescence, human, monkey and dog samples were incubated after immunolabeling for 40 minutes in CuSO₄ solution and on human samples we also applied overnight light treatment previous to the fluorescent labeling.

T1: Primary antibodies

Target	Producer	Cat. No.	Dilution	Lot
CR	Swant	CR6B3	1:5000	010399
ChAT	Merck Millipore	AB144P	1:500	3520940
DARPP-32	Abcam	ab220808	1:1000	1074740-1
nNOS	Abcam	ab1376	1:1000	not provided
PV	Abcam	ab277625	1:1000	GR33711806-5

T2: Secondary antibodies

Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG	Jackson ImmunoResearch	1:1000	711-545-152
CF®568-Donkey AntiMouse IgG	Biotium	1:800	20105
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647	Invitrogen	1:800	A-21447

T3: Other

DAPI	Calbiochem	1:1000	508741
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Microscopy and post-processing

For confocal microscopy, slices were mounted on glass slides in ProLong™ Diamond Antifade Mountant and imaged with a Nikon A1R confocal laser-scanning microscope. All microscopic images are representative images of at least three biologically independent experiments. A pretrained Stardist model was implemented in QuPath^{8,9} for nuclear segmentation of MSNs and granule cells in 60x confocal images of DAPI stained nuclei from the NAcc shell and major Ioc, respectively. Nuclear segmentation was manually corrected in QuPath as necessary. NIS-Elements AR 5.21.01 (Nikon), QuPath 0.5.1, and Adobe Illustrator 2025 programs were used for the post-processing of confocal images.

Statistics and reproducibility

The sample size (“n”) in Figure legends refers to the number of independent experiments (biological replicates). GraphPad Prism software was used for graph construction and statistical comparisons. The statistical tests used in each experiment are described in their respective Figure legend. For the Stardist-assisted nuclear segmentation, the nuclear area and density were normalized to the average values obtained from MSNs in the same mouse or human subject. One sample T-test with a hypothetical mean of 1.000, corresponding to the nuclear area and density values of the NAcc shell, was used to assess significance.

Results

The principal cell type of the loC is the small granule cell (GC)^{10, 11, 12} and the dense aggregation of GC somas forms the “granular subregion” of the loC. As our group has previously shown, GCs have a unique morphology, which is closely linked to the subregions of the loC complex. The dendrites of these cells extend throughout the granular subregion, while their axons are confined to the cell-poor “hilar subregion”. Consequently, they do not form long-range projections. Importantly, GCs do not express the characteristic marker protein of striatal medium spiny neurons (MSNs), dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32). Hence it is an effective negative marker of the loC within the striatum, allowing us to identify the “island-like” structures in coronal brain slices alongside the widely known nuclear marker, 4',6-diamidino-2-phenylindole (DAPI) (Fig. 1).

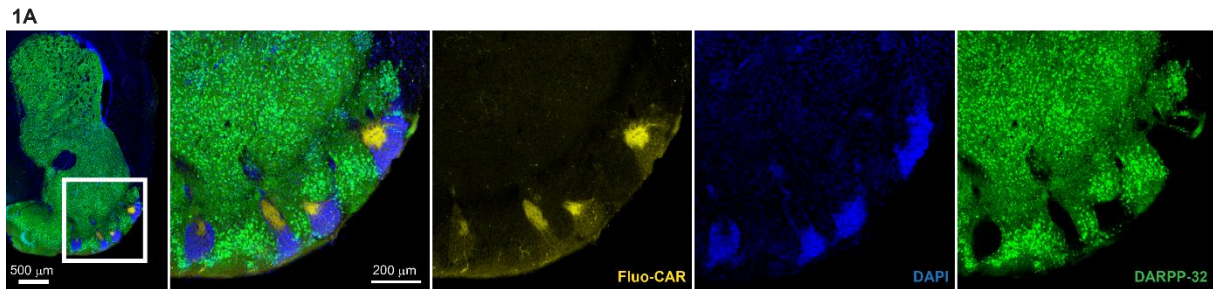


Fig. 1: Fluo-CAR binding in the hilar subregion of the loC in mouse brain tissue

A The loC granular subregion is visualized by DAPI nuclear labeling (blue), the loC hilar subregion is outlined by Fluo-CAR binding (yellow). Both granular and hilar subregions are negative for DARPP-32 labeling (green).

The loC is considered to be an evolutionary conserved anatomical structure¹³, but its presence or organization in the human brain has not yet been clearly established. To address this question, I performed fluorescent immunolabelling experiments using DARPP-32-targeting antibodies on mouse and human samples. I found that the loC appears as distinct cell-dense DARPP-32⁻ islands in both mouse and human brain sections (Fig 2a, 2c).

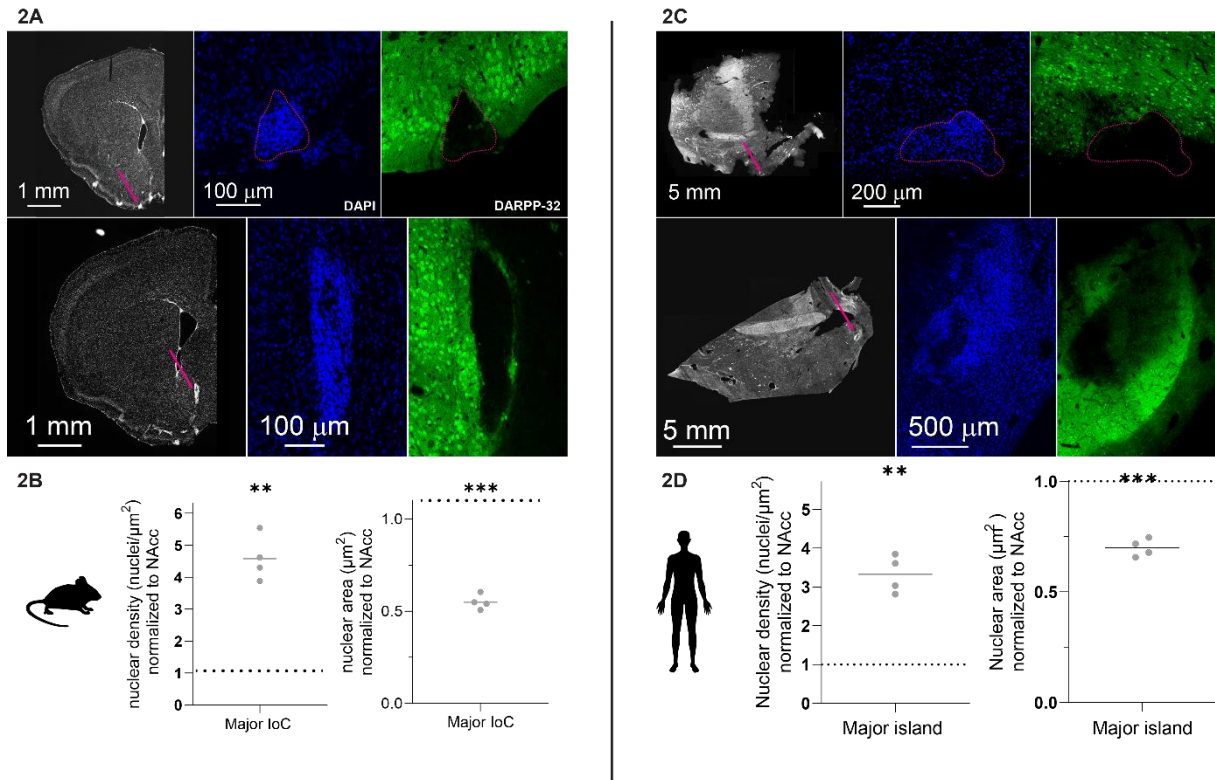


Fig. 2: The IoC has similar morphological and cellular features in both human and rodent brain

A, C Confocal, 20x magnification images of DAPI (blue) and DARPP-32 (green) labelings show that the IoC appears as a cell dense structure in coronal sections in both species. A low magnification overview image of DAPI (left on each panel) is for reference, *locating the exact position, where the 20x images were taken*

B, D Quantification of the islands' nuclear area (right on each panel) and density (left on each panel) normalized to the Nucleus Accumbens (NAcc) in both species, using a Stardist assisted nuclear segmentation model (further information under Materials and methods).

Statistical evaluations were performed by one sample T-tests **B** Number of animals: $n=4$, total number of cells: 2770, mouse nuclear density: $P=0.0002$; **D** Number of samples $n=4$ (two samples from two subjects each), total number of cells: 2770, human nuclear density: $P=0.0003$

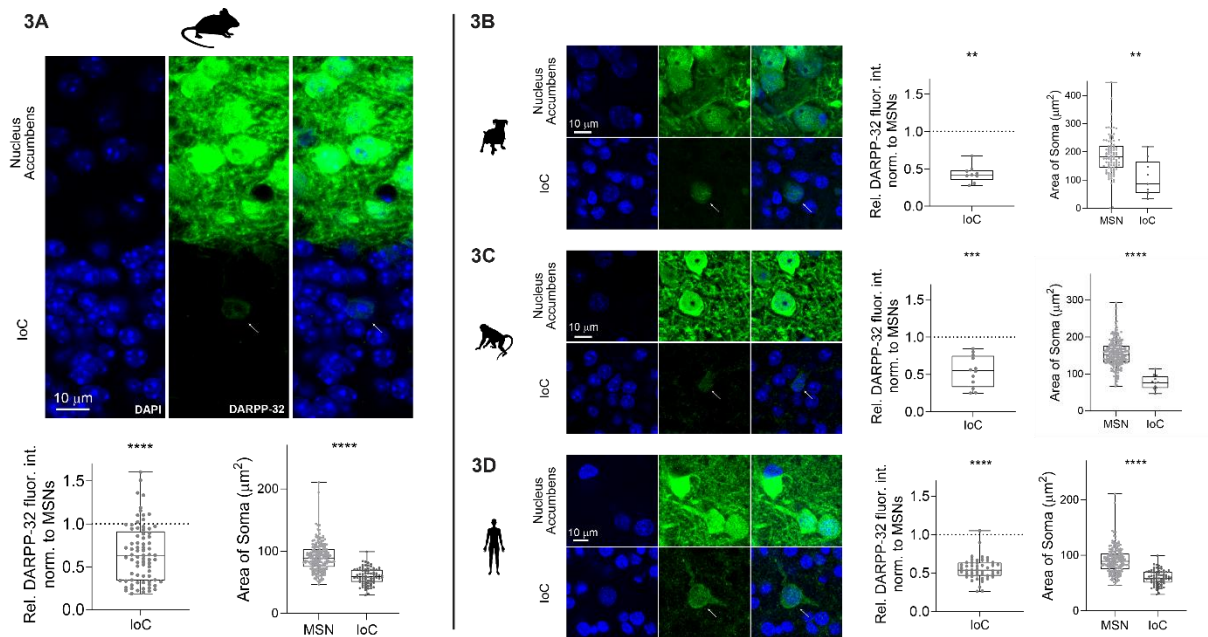


Fig. 3: Small DARPP-32⁺ cells are embedded into the GC cluster

A Confocal, 60x magnification images of low DARPP-32 expression in mouse loC GCs. Quantifications show the relative fluorescent intensity of DARPP-32⁺ cells normalized to MSNs in the NAcc (left), and the area of MSN somas compared to the somas of GCs (right) **B** Confocal, 60x magnification images and quantifications of DARPP-32⁺ cells shown in dog (*Canis Familiaris*) **C** Confocal, 60x magnification images and quantifications of DARPP-32⁺ cells shown in monkey (*Macaca Mulatta*) **D** Confocal, 60x magnification images and quantifications of DARPP-32⁺ cells shown in human (*Homo Sapiens*) tissues.

Statistical evaluation for the area of the soma was performed by two-tailed Mann-Whitney test (A: n=4, p<0.0001, analyzed cell number: 264; B: n=2, p=0.0014, anal. cell number: 112; C: n=2, p<0.0001, anal. cell number: 244; D: n=2, p<0.0001, anal. cell number: 287). Statistical evaluation for the fluorescent intensity (norm. to the NAcc) of the was performed by one sample Wilcoxon test (A: n=4, p<0.0001, analyzed cell number: 77; B: n=2, p=0.0039, anal. cell number: 9; C: n=2, p<0.0001, anal. cell number: 12; D: n=2, p<0.0001, anal. cell number: 34).

To further analyze these structures and quantify their appearance, we systematically compared their cellular density and nuclear size. For relative measurements, we compared these cells to those in the Nucleus Accumbens (NAcc). To enable high-throughput analysis, we applied Stardist (Fig. 2b, 2d), a deep learning-based model for automatic cell nucleus annotation. We found that interestingly similar to the mouse brain, the loC in other species exhibits substantially high cell density, composed of cells with small nuclei. Taken together, our findings suggest that the loC islands are located in the human ventral striatum at sites corresponding to those in the mouse brain and exhibit similar overall morphologies.

The precise cellular composition of the loC has remained unexplored even in rodent models. Therefore, we aimed to improve our knowledge about the neuronal subtypes that are present in the loC complex. Interestingly, after thorough investigation of our immunohistochemical sections revealed that even though the loC is primarily composed of DARPP-32⁻ GCs, it also includes DARPP-32⁺ neurons, embedded into the GC cluster. These cells are significantly smaller than DARPP-32⁺ MSNs in the NAcc (Fig. 3A, bottom left panel) and display lower cytoplasmic DARPP-32⁺ expression (Fig. 3A, bottom right panel). To expand our observations, we used DARPP-32 immunolabeling on tissue sections that were available to us from other species, including dogs (*Canis Familiaris*) and monkeys (*Macaca Mulatta*). Our quantitative analysis showed that this cell population is present in the loC of all the species we studied (Fig. 3B-D).

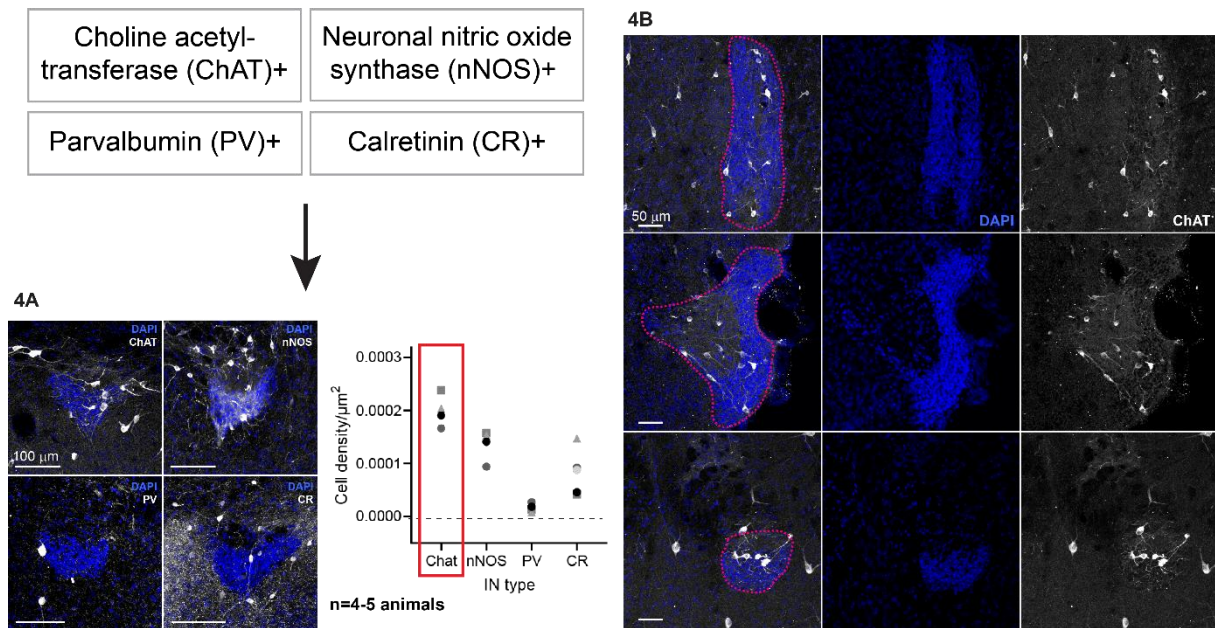


Fig. 4: The four main types of striatal IN markers are present in the mouse loC

A Confocal, 20x magnification images (left) of striatal IN-markers (white) alongside DAPI (blue) and their quantifications (right) show the density of IN-like cells within the loC **B** Confocal, 20x magnification images show the remarkable enrichment of the ChAT⁺ cells in all loC subregions

To further explore the cellular heterogeneity within the loC, we examined the types of interneuron-like (IN-like) cells that the loC may contain. We performed a series of fluorescent labelling experiments using all key neurochemical markers of striatal interneurons^{14, 15, 16}. Choline acetyl-transferase (ChAT), neuronal nitric oxide synthase (nNOS), parvalbumin (PV), and calretinin (CR) are key markers used to differentiate functionally distinct striatal interneurons (INs). First, we quantified the density of each marker throughout the mouse loC complex. We found evidence that cells expressing each of these interneuron markers are present in the loC (Fig. 4a). Notably, we discovered a surprising enrichment of ChAT⁺ neurons across all loC subregions (Fig. 4b). Co-labeling of ChAT and nNOS revealed that ChAT⁺ and nNOS⁺ cells represent partially overlapping populations, which is unlike in other striatal regions¹⁷. Our labelings indicate a complex cellular heterogeneity within the loC and reveal a significant population of ChAT⁺ neurons, which themselves exhibit heterogeneity.

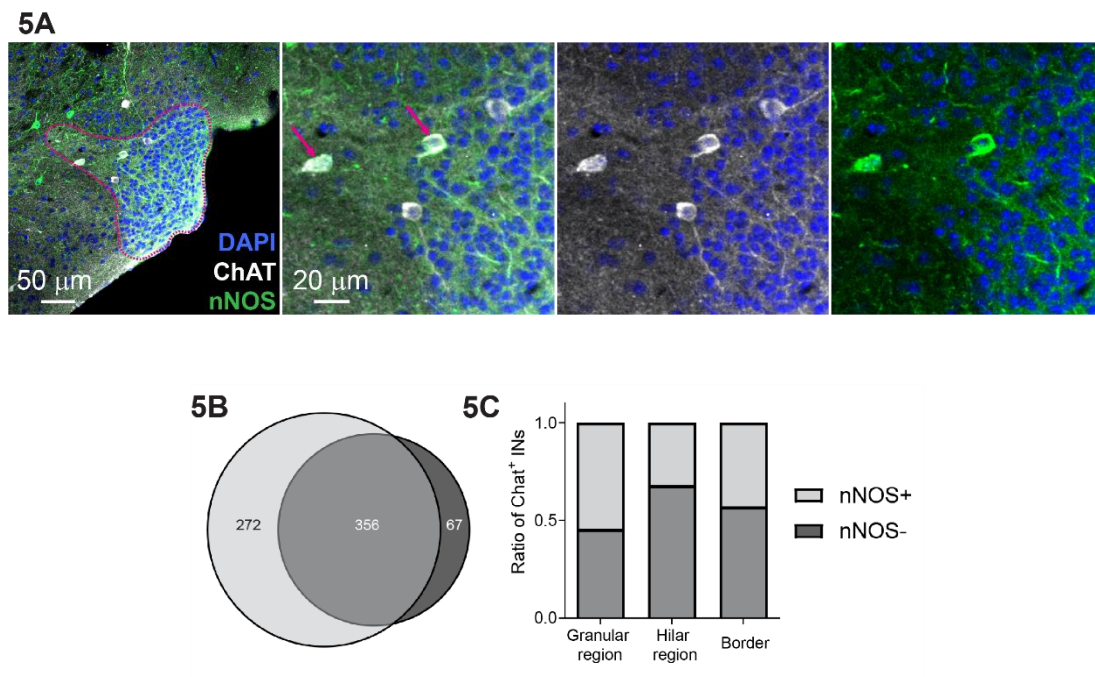


Fig. 5: ChAT⁺ and nNOS⁺ interneuron-like cells represent partly overlapping populations

A Confocal images of the two partly overlapping populations, arrows highlighting the double positive cells

B Venn-diagram showing the ratio of ChAT⁺, ChAT/nNOS double positive and nNOS⁺ cells

C Distribution of ChAT⁺ cells within the islands (Granular/Hilar/Border subregion) (695 cells analyzed)

Since the prominent density of ChAT⁺ cells was evident in the mouse IoC, we investigated whether this phenomenon is an evolutionarily conserved feature. ChAT immunolabeling on sections from dogs, monkeys, and humans revealed a remarkable enrichment of cholinergic neurons in the IoC across all studied species (Fig. 6). These findings highlight a conserved cellular composition of the IoC and suggest an important connection of GCs to the cholinergic system.

A comprehensive anatomical description of the cellular heterogeneity within the IoC should include more than just data about neurons, as it is equally important to investigate the non-neuronal components. Although astrocytes and microglia, have been frequently implicated in affective disorders^{18, 19}, our analysis did not reveal any significant differences in the glial

composition of the loC compared to neighboring regions. Both astrocytes and microglia are indeed present in the loC complex (Supp. Fig. 1).

Our findings may serve as a base for future studies addressing the physiological role of this structure, and these results could have major implications not only for affective disorders, but potentially for addiction studies as well.

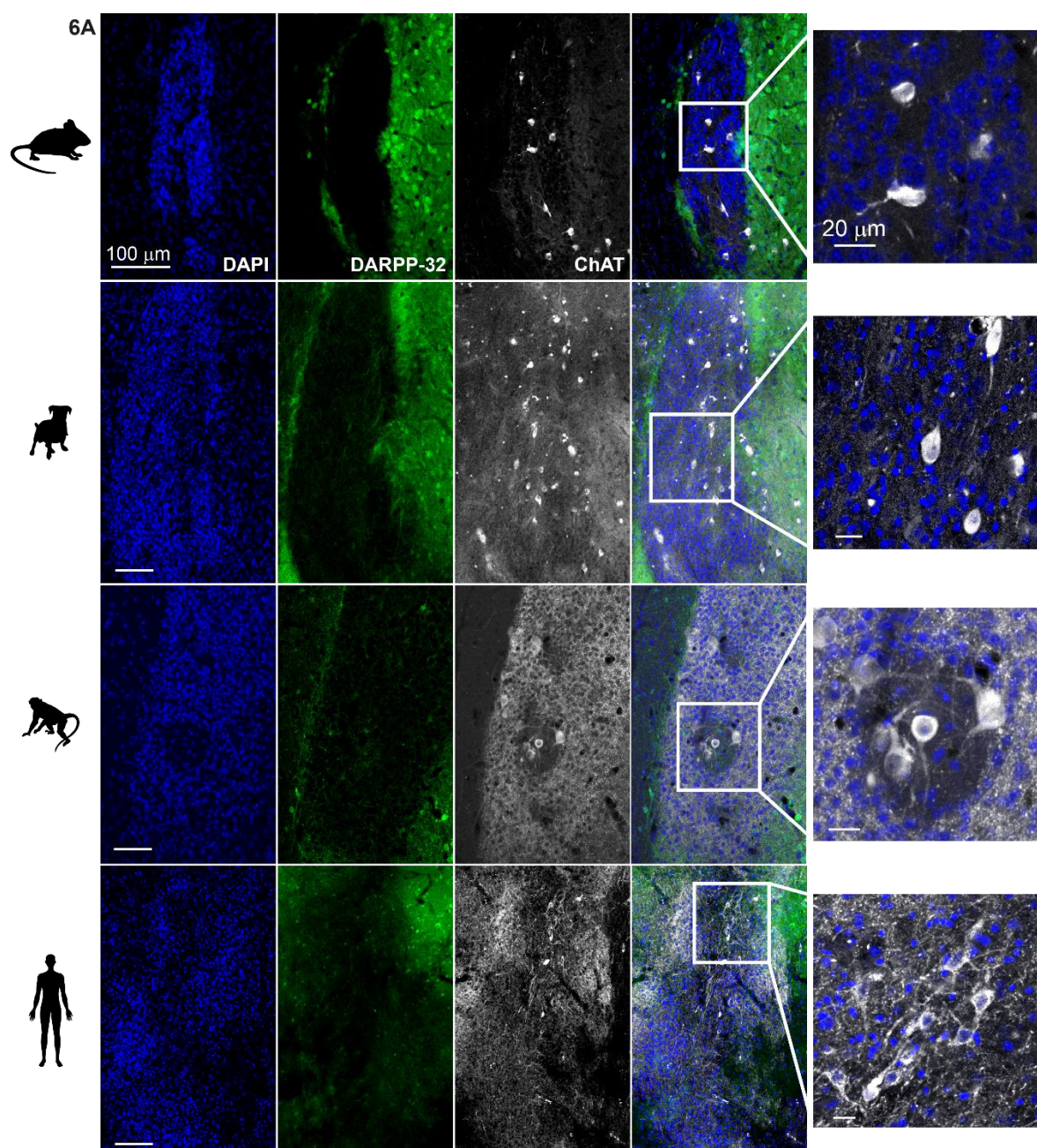


Fig. 6: ChAT⁺ cell enrichment in the loC is evolutionary conserved

A Confocal images show DAPI (blue), DARPP-32 (green), and ChAT (white) labeling in mouse, dog, monkey, and human brain tissue.

Discussion

Although the presence of the “islands” in the basal forebrain was first described over 100 years ago, the Islands of Calleja (IoC) remain one of the most understudied brain regions. One reason for the lack of anatomical and functional data about the human IoC is its earlier classification as part of the olfactory tubercle. Since humans are considered microsmatic mammals with a reduced olfactory tubercle, the existence of the IoC in the human brain has largely been ignored. However, recent rodent studies have repeatedly linked the IoC to affective disorders such as bipolar depression and schizophrenia, and our group has shown that IoC granule cells (GCs) are a key target of the antipsychotic drug, cariprazine.

Island-like structures have been reported in earlier low-resolution studies of human brain samples under various nomenclature and anatomical positions. However, these studies relied on nonspecific markers and subjective grouping of cells as “islands.” Consequently, the entire IoC complex has not been captured, as islands may appear as small clusters of cells in single coronal sections. These “islands” are easily missed by prior methods. Additionally, the potential presence of other cell-dense basal forebrain structures (e.g., interface islands in non-human primates)²⁰ might have led to their misidentification. A comprehensive analysis of the IoC is therefore essential to clarify its anatomical integration within the complex human basal forebrain.

Here, we apply immunohistochemistry and quantitative image analysis for the first time to unambiguously identify IoC islands in the human basal forebrain and compare their cellular composition directly with that of the mouse. Our preliminary data reveal that the IoC displays similar morphological and cellular features in both humans and mice. In both species, nearly all densely packed granule cells (GCs) are negative for DARPP-32, with a small subpopulation of DARPP-32⁺ neurons embedded within the GC clusters. Interestingly, these DARPP-32⁺ neurons exhibit significantly lower expression levels of this marker protein. This unique subpopulation of DARPP-32⁺ neurons within the IoC has not been reported before and may have a distinct physiological and pathophysiological role related to the activity of IoC GCs.

Additionally, we provide the first quantitative evidence of cells within the loC expressing markers of distinct interneuron types found in the striatum, suggesting that loC circuitry is fine-tuned by local regulation. Importantly, all our investigations were performed across the entire rostrocaudal and mediolateral extent of the loC, capturing the properties of the entire complex. This represents the first anatomical study to comprehensively analyze the loC in this way.

Since distinct striatal interneuron classes are associated with specialized functions and various diseases, selectively investigating their roles within the loC under different perturbations represents an intriguing future direction. Notably, we have identified a remarkable number of ChAT⁺ (choline acetyltransferase-positive) interneuron-like cells within the loC. Preliminary co-labeling analyses suggest that these cells represent a heterogeneous population. Striatal cholinergic neurons have been implicated in key basal ganglia functions, including reward-related behavior, learning, and motor control²¹. Additionally, GABA and glutamate co-releasing cholinergic interneurons have been identified in the striatum using genetic and immunolabeling approaches²², with distinct roles reported in Parkinson's disease. In our study, nNOS/ChAT co-labeling identified two separate clusters of cholinergic neurons within the loC, supporting their heterogeneity in the loC as well. Future experiments may explore how the subgroups of cholinergic neurons within the loC and other ventral striatal structures overlap.

Although our microscopic images implicate that many cholinergic fibers from these cells remain restricted to the loC territory, further analysis is required to precisely characterize their morphology and draw more direct conclusions about their functional integration into the loC circuitry. The dense presence of cholinergic fibers, and the resulting potentially high local acetylcholine concentrations, aligns well with the observation that loC granule cells (GCs) express high levels of M4 muscarinic acetylcholine receptors. This is particularly interesting, as a recent blockbuster drug, xanomeline, exerts its effects via M4 muscarinic acetylcholine receptors²³. This represents the second clinical drug, in addition to cariprazine, which may mediate unique antipsychotic effects through the loC. However, the functional role of loC GCs and their involvement in these pharmacological effects need to be validated through future studies employing cell-type-specific knockout technologies.

A key technical challenge in our study arises from the quality of post-mortem human tissue, as the ventral striatal region where the loC is located, is an end-arterial region lacking anastomoses. This anatomical characteristic makes the region particularly vulnerable to poor fixation, which can substantially hinder immunohistochemistry, introduce variability, and compromise data quality. To address this, we are making extensive efforts to optimize post-mortem fixation techniques, including improved perfusion protocols and the post-fixation of tissue blocks, and the careful selection of well-preserved samples to ensure the reliability of our analyses.

From a functional perspective, a limitation of our immunohistochemical findings is that they primarily suggest functional connections without confirming direct synaptic relationships. For instance, a systematic characterization of the synaptic interactions between granule cells (GCs) and loC interneurons is beyond the scope of this study. Electrophysiological experiments are necessary to analyze these interactions functionally and validate their roles within the loC circuitry. How can our study facilitate future translational research beyond providing specific anatomical data? We believe that our results can lay the groundwork for targeted rodent studies by identifying key conserved features of the loC. For instance, our analysis can guide cell-type-specific manipulations or provide a set of conserved immunohistochemical markers to prioritize in future studies. Additionally, our findings could guide post-hoc anatomical investigations of samples from non-healthy individuals with documented affective disorders. Such analysis may help to uncover potential loC-related pathophysiological mechanisms.

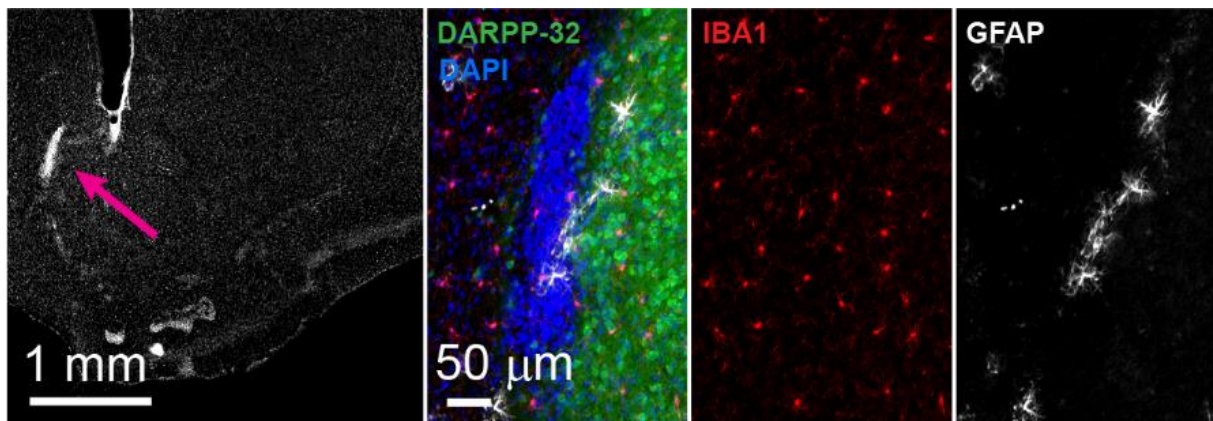
Our future aim in the research project pipeline is to create a 3D map of the loC in the human brain. We are currently working on incorporating additional specific loC immunomarkers, alongside DARPP-32, to ensure we can clearly delineate both the cell-dense granular and cell-poor hilar subregions of the human loC from neighboring regions. A future comprehensive map will facilitate high-resolution functional imaging of the human loC. Additionally, it can guide therapeutic interventions like deep brain stimulation, which are already used in adjacent regions, such as the Nucleus Accumbens, for the treatment of conditions like treatment-resistant depression.

To summarize, the conserved molecular and anatomical properties of the loC circuitry suggest similar physiological and pathophysiological significance of this brain region in rodents and humans. Our findings provide a foundational framework for future loC studies and may help to uncover the exact role of this enigmatic brain region.

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Supplementary information



Supplementary Figure 1. Glial composition of the IoC

Low magnification overview image, indicating the location of the island on a coronal mouse brain section (left). Higher magnification image of a fluorescent labeling, using a microglia marker, the ionized calcium-binding adapter molecule 1 (IBA1, in red), and an astrocyte marker, glial fibrillary acidic protein (GFAP, in white), alongside DAPI (blue), DARPP-32 (green).

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