Afferent islands are larger than μ-opioid receptor patch in striatum of rat pups

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Dopamine afferent islands were observed in rodent caudate-putamen only during development, whereas patches with intense μ-opioid receptor (MOR) immunoreactivity were seen throughout the life. We performed direct comparison between MOR patches and dopamine islands in the caudate-putamen of rat pups, by double immunofluorescence labeling for MOR and tyrosine hydroxylase. MOR patches were included in dopamine islands at postnatal day (P) 0 to P8, although the patches occupied the same region as the islands at P12–16. Furthermore, the regions of glutamatergic afferents with intense vesicular glutamate transporter 1 and vesicular glutamate transporter 2 immunoreactivities well corresponded to those of dopamine islands at P4. These results suggest that the striatal 'afferent islands' are larger than MOR patches in the early postnatal life. ***NeuroReport***

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Introduction

Mammalian caudate-putamen (CPu) shows mosaic organization of two anatomically distinct compartments. The mosaic organization was first demonstrated as 'dopamine islands' in the immature CPu of rat by histofluorescence [1] and then by immunohistochemistry for tyrosine hydroxylase (TH) [2]. Thus, dopaminergic axons innervated the patchy 'islands' more densely than surrounding 'matrix' in rat CPu transiently during the first 2 weeks after birth [3].

In contrast, in mature rat CPu, μ-opioid receptor (MOR)-enriched 'patches' (or 'striosomes') and surrounding 'matrix' were observed by receptor binding [4], in-situ hybridization for mRNA [5–7], and immunohistochemistry for receptor protein [8–11]. Patches of MOR immunoreactivity were also observed throughout postnatal development [12]. In addition, we recently reported that vesicular glutamate transporter 1 (VGLUT1) and VGLUT2 immunoreactivities, which were markers of glutamatergic axon terminals of cortical and thalamic origins, respectively, in CPu [13], showed island-like profiles in association with dopamine islands in mouse pups [14].

However, relationship between dopamine islands and MOR patches in immature CPu has been unsettled so far, although correspondence of acetylcholinesterase-poor striosomes with dopamine islands has been established [2]. In this study, we performed double immunofluorescence labeling for MOR and TH on the CPu of rat pups to study relationship between MOR patches and dopamine islands. Furthermore, we also compared boundaries of intensely VGLUT1-immunoreactive or VGLUT2-immunoreactive 'glutamate islands' with those of dopamine islands and MOR patches.

Methods

Animals and tissue

The experiments were conducted in accordance with the rules of animal care of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. Pregnant Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The day of birth was defined as postnatal day (P) 0. A total of 18 rats (three for each age) of P0, P4, P8, P12, P16, and P22 were used in this study. All efforts were made to minimize animal suffering and the number of animals used.

Rats were deeply anesthetized by cooling on ice for P0–P4 or with intraperitoneal injection of chloral hydrate (35 mg/100 g body weight) for P8–P22. The rats were transcardially perfused with 5 mM phosphate-buffered 0.9% saline (PBS; pH 7.3), and then with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.3). The brains were removed and postfixed in the same fixative overnight at 4°C. After cryoprotection with 30% sucrose in PBS, brains were cut into 50-μm-thick coronal sections on a sliding microtome.

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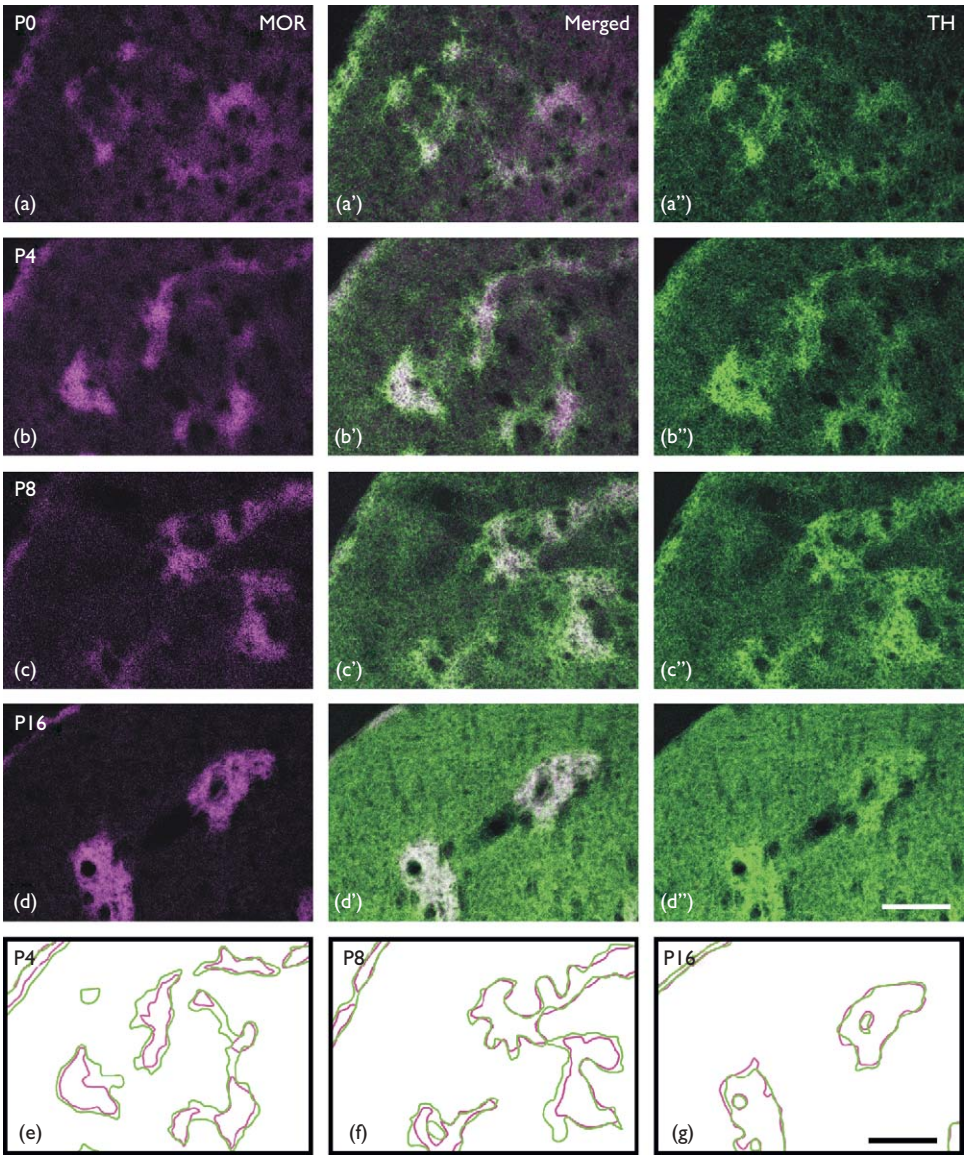
Immunofluorescence labeling

Multiple immunofluorescence labeling was performed as described earlier [14]. The following primary antibodies were used: (i) anti-MOR (2 μg/ml, guinea pig) [9,11] and anti-TH (1/3000, mouse; Chemicon, Temecula, California, USA); (ii) anti-MOR (2 μg/ml, guinea pig), anti-TH (1/3000, mouse), and either anti-VGLUT1 or VGLUT2 (2 μg/ml, rabbit) [15]. Their immunoreactivities were detected, respectively, by secondary antibodies (10 μg/ml each): (i) anti-mouse (goat, labeled with Alexa Fluor 488; Molecular Probes, Eugene, Oregon, USA), and anti-guinea pig (donkey, Cy3; Chemicon); (ii) anti-rabbit (goat, Alexa Fluor 488; Molecular Probes), anti-guinea pig (donkey, Cy3; Chemicon), and anti-mouse (goat, Alexa Fluor 647; Molecular Probes). Digital images of labeled sections were obtained with a confocal microscope (LSM5 Pascal; Zeiss, Oberkochen, Germany).

Tracing

Thresholding of each color channel based on entropy of grayscale histogram [16] were applied to the digital images automatically with ImageJ software (National

Fig. 1



Double immunofluorescence labeling for μ-opioid receptor (MOR; magenta) and tyrosine hydroxylase (TH; green) on sections containing the caudate-putamen (CPu) of postnatal day (P) 0 (a–a″), P4 (b–b″), P8 (c–c″), and P16 (d–d″) rat pups. Images of the dorsolateral part of CPu are presented. Panels e–g display traces of boundaries of intensely MOR-immunoreactive 'patches' (magenta) and highly TH-immunoreactive 'dopamine islands' (green) from panels b–d″. Note that dopamine islands are larger than MOR-immunoreactive patches at P0–P8 (a′, b′, c′, e, f), and that boundaries of dopamine islands showed a good correspondence to those of MOR patches at P16 (d′, g). Scale bar = 200 μm.

Institute for Health, Bethesda, Maryland, USA) and a plug-in 'maximum entropy thresholding' to make black-and-white binary images. Boundaries of MOR patches and dopamine islands therein were traced manually on a software Canvas X (ACD systems, Saanichton, British Columbia, Canada).

Results

To directly examine relationship of MOR patches and dopamine islands in CPu, we conducted double immunofluorescence labeling of MOR and TH on specimens of rat pups (Fig. 1). Intensely MOR-immunoreactive patches well overlapped the core of highly TH-immunoreactive dopamine islands (Fig. 1a′–d′). However, the periphery of dopamine islands was characterized by the paucity of MOR immunoreactivity from P0 to P8, most notably at P4 (Fig. 1e and f). Dopamine islands were thus often larger than MOR patches. Similarly, the partial mismatch was also observed at the subcallosal streak, the edge of CPu beneath the cerebral white matter (Fig. 1e and f). By contrast, dopamine islands were well in register with MOR patches at P12 (not shown) and P16 (Fig. 1d′ and g). At P22, dopamine islands were no longer observed in TH-immunolabeled sections; TH immunoreactivity was distributed homogeneously throughout the CPu.

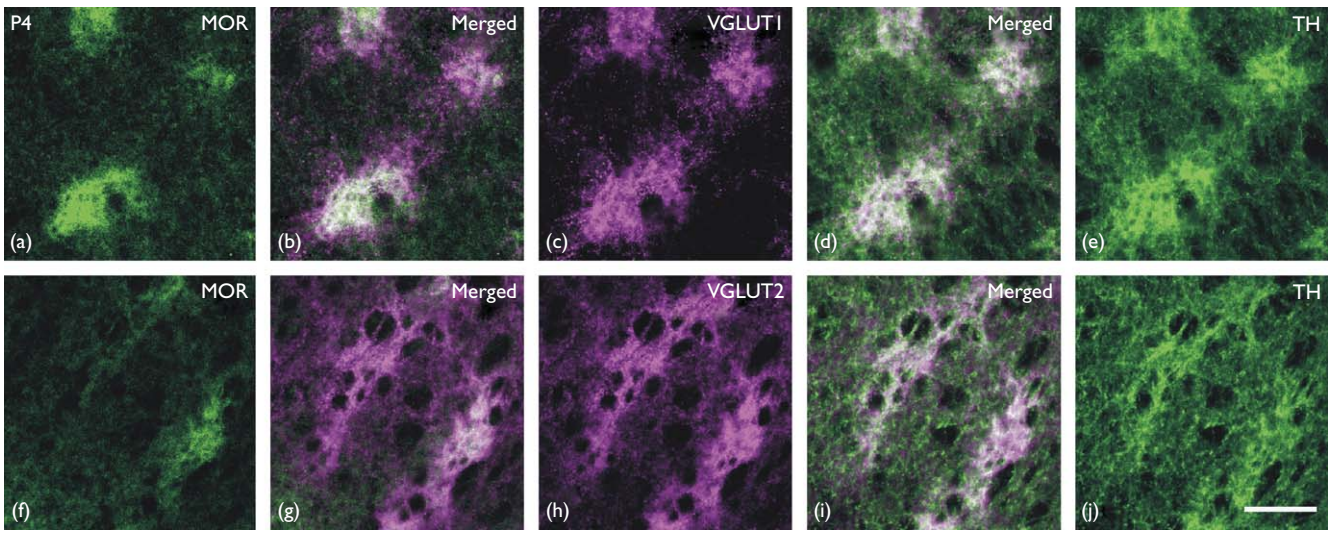
We next performed triple immunofluorescence labeling of MOR, TH, and either VGLUT1 or VGLUT2 to compare the boundaries of intensely VGLUT-immunoreactive 'glutamate islands' with those of MOR patches and dopamine islands on P4 specimens (Fig. 2). VGLUT1-immunoreactive islands overlapped dopamine islands and MOR patches in CPu (Fig. 2a–e). The boundaries of VGLUT1 islands were mostly in register with those of dopamine islands (Fig. 2d), whereas VGLUT1 islands were often larger than MOR patches (Fig. 2b). Likewise, intensely VGLUT2-immunoreactive islands overlapped dopamine islands and MOR patches at the ventromedial part of CPu (Fig. 2f–j). When we compared their boundaries, VGLUT2 islands were in register with dopamine islands (Fig. 2i), but often larger than MOR patches (Fig. 2g). At the dorsolateral part, however, VGLUT2 immunoreactivity was weak and did not show such islands clearly. Thus, intensely VGLUT-immunoreactive 'glutamate islands' better overlapped dopamine islands than MOR patches.

Discussion

Boundaries of 'glutamate islands' of intense VGLUT1 or VGLUT2 immunoreactivities well corresponded to those of dopamine islands in the CPu of rat pups, as reported earlier in mice [14]. In mature CPu, VGLUT1 and VGLUT2 immunoreactivities are assumed to represent corticostriatal and thalamostriatal glutamatergic axon terminals, respectively [13]. Thus, three major afferents to CPu, that is, nigral dopaminergic afferent, and cortical and thalamic glutamatergic afferents, may share a common boundary of mosaic organization to form 'afferent islands' in immature CPu.

Despite a clear correspondence of the core of afferent islands with MOR patches, the periphery of afferent

Fig. 2



Triple labeling for μ-opioid receptor (MOR; green; a, b, f, g), tyrosine hydroxylase (TH; green; d, e, i, j), and either vesicular glutamate transporter 1 (VGLUT1; magenta; b–d) or VGLUT2 (magenta; g–i) on sections containing caudate-putamen (CPu) of postnatal day (P) 4 rat pups. Panels a–e are taken from the dorsolateral part, and f–j from the ventromedial part of CPu. Intensely VGLUT-immunoreactive islands overlapped MOR patches and dopamine islands. However, note that the size of VGLUT1-immunoreactive and VGLUT2-immunoreactive islands is in register with that of dopamine islands (d, i), but often larger than that of MOR patches (b, g). Scale bar = 100 μm.

islands was almost devoid of MOR immunoreactivity. This spatial mismatch could be explained, if our antibody cannot recognize perinatal period-specific MOR variants that are expressed at the periphery. Developmental switch of MOR variants were suggested by the findings that [^{125}I]β-endorphin-crosslinked opioid receptors in P1 rat brain homogenate migrated with a 55 kDa band alone despite competition binding data indicating the presence of MOR, whereas that in adults migrated with a MOR-selective 65 kDa band predominantly [17], and that ligand selectivity of MOR in P1 rat striatum was lower than in adults [18]. In fact, six alternatively spliced variants of rat MOR with differential ligand-binding characteristics have been described so far [19]. However, our antibody was raised against C-terminal 30 amino acids of rat MOR1 [9]. The amino acids are encoded by portions of two exons of MOR gene; one is specific for rat MOR1, whereas the other is a common exon for all six variants [9,11,19]. As the antibody bound with both two peptide fragments corresponding to each exon [11], we consider that the present results may account for the majority, if not all, of MOR immunoreactivity in rat pups.

Immunoelectron microscopic analysis reported that MOR immunoreactivity in rat neonatal CPu was highly localized to perikaryal membranous structure, in sharp contrast to the preferential localization to dendritic plasma membrane in adults [12]. In line with this notion, binding assay of [^{3}H][D-Ala^{2}, Me-Phe^{4}, Gly-o1^{5}]enkephalin showed that 43% of MOR binding site in P1 rat forebrain was localized to endoplasmic reticulum and Golgi apparatus, whereas 84% in adults was on plasma membrane [20]. Thus, subcellular localization of MOR in neonatal striatal neurons was different from that of mature neurons. Given the predominant localization of MOR to perikarya in neonates, the periphery of afferent islands may be occupied by the MOR-negative dendrites extended from the MOR-expressing patch neurons. If this is the case, the boundary of MOR patches expands to that of the afferent islands during later development. The fact that afferent fibers develop before the MOR expression may propose a possibility that some afferent activity induce the gene expression including MOR. The interaction of afferent axons and dendrites of patch neurons needs to be clarified in future studies to advance understanding of the formation of mosaic organization in CPu.

In neonatal rats, intrastriatal injection of retrograde tracer resulted in labeling of neurons in the intralaminar thalamic nuclei [21]. Those nuclei thus seem to originate from putative thalamostriatal axon terminals immunopositive for VGLUT2 in the afferent islands. In contrast, given anterograde tracing experiments in rat pups [22], the prelimbic cortex could be a likely origin of VGLUT1-loaded corticostriatal axon terminals in the islands. Thus, emotional information from the limbic system and information from the intralaminar thalamic nuclei could converge through the channel of afferent islands to flow into the immature basal ganglia. Striatonigral projection neurons thereof could inhibit activity of nigrostriatal dopaminergic neurons even at P1 [23]. The inhibition of nigro-striosomal dopamine signal might in turn modulate characteristics of glutamatergic synapses in the afferent islands. Thus, the patch/striosome system could perform dopamine-mediated learning before development of the matrix system in the basal ganglia.

Conclusion

In this study, we showed that the core, but not periphery, of dopamine islands overlapped MOR patches in the neonatal rat CPu from P0 to P8. In P12–P16 rats, dopamine islands were in register with MOR patches, indicating their mismatch was a transient event in early postnatal period. We further reported that dopamine islands and 'glutamate islands' visualized with VGLUT1 and VGLUT2 immunoreactivities share the common boundaries in P4 rat CPu and together form 'afferent islands'.

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