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Cannabinoid Receptor Expression at the MNTB-LSO Synapse in Developing Rats

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Abstract

The organization of developing auditory circuits depends on the elimination of aberrant connections and strengthening of appropriate ones. Endocannabinoid mediated plasticity is a proposed mechanism for this refinement. Here we investigated for the anatomical presence of cannabinoid receptors (CB1R) in the lateral superior olive (LSO) and medial nucleus of the trapezoid body (MNTB) of developing rats. We found that CB1R is present within the LSO and that it colocalized with vesicular glutamate transporter (VGLUT3), a presynaptic marker for MNTB terminals. Both before (P5) and around hearing onset (P12), the expression levels of CB1R were higher in the lateral limb of the LSO than in the medial limb. These results suggest that endocannabinoid signaling can modulate the strength of the developing MNTB-LSO synapse.

Keywords

Lateral Superior Olive (LSO); Medial nucleus of trapezoid body (MNTB); Endocannabinoids; Cannabinoid receptors (CB1R)

Introduction

In many neuronal systems, precision of synaptic circuits is achieved by activity-dependent refinement of developing connections, involving the elimination of aberrant projections and the strengthening of remaining inputs [9, 16]. This reorganization is essential for establishing topography in sensory systems, the central representation of spatial relationships of sensory receptors. Reorganization or developmental increase in tonotopic organization also is present in the central auditory system both on cortical and subcortical levels [6, 8, 10, 12, 21].

The lateral superior olive (LSO) is a nucleus relevant to encode interaural intensity differences which are major cues for determining the horizontal direction of incoming sounds. LSO neurons integrate excitatory inputs from the ipsilateral ear and inhibitory inputs from the contralateral ear [25]. The excitatory inputs are carried by glutamatergic fibers from the cochlear nucleus, while the inhibitory inputs arrive from glycinergic fibers of the medial nucleus of the trapezoid body (MNTB). These converging bilateral inputs are

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tonotopically arranged so that the same sound frequency excites and inhibits single LSO neurons.

During development, the precise organization of synaptic circuitry in the LSO emerges via functional and structural reorganization of the MNTB-LSO pathway. Before hearing onset, single LSO neurons lose approximately 75% of their initial MNTB inputs and synaptic conductance of the existing inputs increases about 12 fold [10, 11]. After hearing onset, structural reorganization takes place in the form of pruning of MNTB axons and LSO dendrites [20, 22]. Axonal and dendritic pruning requires normal neuronal activity as it is impaired by cochlear ablation or chronic blockade of glycinergic transmission [22, 23].

The mechanisms that underlie tonotopic refinement of the MNTB-LSO pathway are incompletely understood. It has been proposed that endocannabinoid signaling is responsible for activity dependent synaptic plasticity [3]. Endocannabinoids are released from postsynaptic terminals and subsequently bind to presynaptic, G protein-coupled, cannabinoid receptors (CB1R) [3, 4]. The activation of CB1R inhibits neurotransmitter release. Thus, endocannabinoids act as retrograde messengers that allow postsynaptic cells to regulate the strength of synaptic inputs.

Endocannabinoid mediated plasticity has been demonstrated in the auditory system. In the dorsal cochlear nucleus (DCN), cartwheel cells can release endocannabinoids in an activity-dependent manner which binds to presynaptic CB1R at glutamatergic inputs leading to long term depression (LTD) [27-29]. In the MNTB, synaptic strength at the calyx of Held is decreased by activation of CB1R which are expressed presynaptically [15]. In the developing inferior colliculus of chickens, activation of CB1R induces LTD, and its expression involves pre- and postsynaptic mechanisms [19].

Here we characterized with immunohistochemistry the expression and anatomical location of CB1R within the developing LSO and MNTB. We also investigated whether the expression of CB1R varies during the period prior to hearing onset, at the time of onset, and in an adult rat. Our results support the hypothesis cannabinoid receptors are present at this synapse which raises the possibility that they may be involved in the tonotopic sharpening of the MNTB-LSO pathway.

Materials and methods

All experimental procedures were in accordance with NIH guidelines and were approved by the IACUC at the University of Pittsburgh. Postnatal day 5 (P5) and 12 (P12) and adult rats were deeply anesthetized with isoflurane and perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde (PFA). Brains were removed and fixed in PFA overnight. Tissues were cryoprotected in 30% sucrose and coronal brainstem slices (50 μ m) were cut with a sliding microtome (Microm HM 430, Thermo Fisher Scientific, Waltham, MA).

Immunohistochemistry was performed on free floating sections. Sections were preincubated in 5% normal goat serum (NGS) in PBS for 2 hours then incubated with an immunopurified rabbit antibody against the C terminus of rat CB1 at 1: 2000 (gift from Dr. Ken Mackie, Indiana University, Bloomington, IN) [29] for 2 hours at room temperature and then 2 days at 4 °C. Tissue sections were then processed using conventional avidin-biotin horseradish peroxidase complex method (ABC; Elite, Vector Laboratories, Burlingame, CA). Sections were then incubated with biotinylated goat anti-rabbit secondary antibody (1:200) for 2 hours and avidin-biotin complex for 2 hours. The specimens were subsequently stained with 0.05% 3,3' diaminobenzidine tetrachloride (DAB, Sigma-Aldrich, St. Louis, MO) for 15 minutes and then with hydrogen peroxide with DAB for 2-3 minutes. Sections were

mounted and coverslipped. For negative controls, sections were incubated with secondary antibody without the primary antibody. Under these conditions no labeling was observed (data not shown).

Quantification of CB1 immunoreactivity was performed by measuring the average absolute gray values (AGV) in regions of interest (ROI, 100 μm by 100 μm) from the medial, central, and lateral limbs of the LSO and from a region just dorsal to the LSO using Image J (version 1.43m; National Institutes of Health, USA). Relative gray values (RGV) were calculated using the following equation [17]:

$$\text{RGV} = 100 - (\text{AGV} / \text{AGV}_{\text{background}} \times 100).$$

While the AGV of the ROI is an objective measurement, the interpretation of the values needed to be taken in context with the nonspecific labeling of the background. In order to address this potential, RGV of each ROI was determined compared to the background. The mean RGV along with standard deviations and standard error of the means from each of the LSO sections were then compared using statistical analysis with paired student's t-test.

For double immunofluorescence labeling, the primary antibody, guinea pig anti-VGLUT3 (Millipore, Temecula, CA) was used. Secondary antibodies were goat anti-rabbit conjugated to Alexa 488 (Molecular Probes, Eugene, OR) and donkey anti-guinea pig conjugated to Cy5 (Jackson ImmunoResearch, West Grove, PA). Fluorescent images were acquired on a confocal microscope (10x, 0.4-NA and 60x, 1.4-NA lens, Olympus, FV-500) with sequential imaging of each channel to avoid bleed-through between channels.

Results

We examined the expression of cannabinoid receptors in the LSO of rats using immunohistochemistry for CB1R. CB1R immunoreactivity was consistently observed in the superior olivary complex of all P5, P12, and adult rats ($n=4$ at each age). As shown in figure 1, immunolabeling was present in the MNTB, LSO, and superior periolivary nucleus (SPON). Labeling in the LSO was weaker than in the MNTB and SPON, but labeling could be clearly and consistently observed under higher magnification (Fig. 1B-D).

Immunoreactivity was present as a diffuse staining in the somata and as punctuate staining around cell bodies and in the neuropil. In P5 and P12 rats, segments of thin, untapered fibers with boutons, some of which encircling cell bodies, were routinely observed (Fig. 1B and D). This suggests that CB1R in the LSO is expressed in axonal fibers and presynaptic boutons. However, in adult rats, labeled fibers were not observed as labeling was restricted to isolated boutons.

Although CB1R labeling was found throughout the entire LSO, expression levels were highest in the lateral limb (low-frequency area) giving rise to a medio-lateral gradient (Fig 2). Quantitative analysis of relative grey values obtained from the lateral, middle and medial limb of the LSO revealed 27% and 65% higher values for lateral areas compared to medial areas in the P5 and P12 rats, respectively (3 sections per animal per age, 4 animals, $p=0.0051$ (P5), $p=0.0097$ (P12), paired t-test). This gradient, however, was absent in the adult rat. To determine whether CB1R is expressed by MNTB axons and their presynaptic boutons we double-immunostained CB1R and vesicular glutamate transporter 3 (VGLUT3), which in the LSO, is localized to MNTB terminals [5]. Consistent with previous studies [2, 5] VGLUT3 immunoreactivity in the P5 rat was strong in the LSO and SPON, outlining their nuclear shapes (Fig 3A-C). At higher magnification, VGLUT3 labeling highly co-localized with CB1R labeling (Fig., 3 D-F) although some punctae labeled only for

VGLUT3 or CB1R. In the MNTB, cell bodies were immunoreactive both for VGLUT3 as well as CB1R whereas axons were only positive for CB1R (Fig. 3G-I). The high degree of co-localization of CB1R labeling with VGLUT3 labeling indicates that in the developing LSO, CB1R are located on pre-synaptic MNTB terminals.

Discussion

Our results demonstrate that CB1R are expressed on MNTB terminals in the LSO of rats during the first postnatal week. In the developing LSO, CB1R immunoreactivity was most prevalent in presumptive axons and boutons and strongly co-localized with VGLUT3. Because VGLUT3 is strongly expressed by MNTB terminals during the first postnatal week we conclude that CB1R are located predominantly on presynaptic MNTB terminals. In accordance with this, MNTB cell bodies were immunoreactive for both VGLUT3 and CB1R, whereas axons in the MNTB were only CB1R positive. The CB1R expression on axons in the MNTB is consistent with previous findings showing the expression of CB1R on afferent axons and presynaptic calyces. [15] In our studies we could not identify calyceal endings based on CB1R immunoreactivity which likely is due to the immature state of pre-calyces at this age [7].

Although most CB1R labeled punctae were positive for VGLUT3, we also observed punctae labeled only for VGLUT3 or CB1R indicating that a fraction of MNTB terminals do not express CB1R. In addition, CB1R may also be expressed on VGLUT3-negative MNTB terminals or on presynaptic inputs arising from the cochlear nucleus. This raises the possibility that CB1R may also modulate glutamatergic synapses in the LSO, a hypothesis which awaits further investigation.

The presence of CB1R labeling in the soma of developing MNTB neurons varies from previous findings which demonstrated that CB1R in the MNTB are restricted to the calyx of Held [15]. This difference likely reflects the age differences of the experimental animals used in both studies. In our study, we used P5 rats, an age when functional silencing of MNTB-LSO synapses takes place and when MNTB-LSO synapses are depolarizing and glutamatergic. In contrast, the previous study examined rats that were P10-14, the age when hearing begins and the calyx has achieved a mature-like morphology [7] and has acquired most of its adult-like physiological properties [24]. In order to further investigate whether this variation in expression is related to the age of the rat, we examined the labeling of CB1R in the P12 rat and observed immunopositive calyces consistent with previous reports [16] (data not shown) although at this age some labeling of MNTB somata was still present.

CB1R expression levels in the LSO showed a gradient along the tonotopic axis such that expression levels were significantly higher in the P5 and P12 rats in the lateral, low-frequency limb than in medial, high-frequency limb. This gradient may reflect variations in the distribution of different morphological cell types in the LSO. For example, bipolar cells with high spontaneous rates of miniature excitatory postsynaptic currents (EPSCs) are mostly prevalent in the medial LSO while multipolar cells with low rates of miniature EPSCs predominate in the lateral LSO [14, 20]. Our data predict that MNTB inputs onto multipolar cells may be under stronger modulation by endocannabinoid signaling than inputs onto bipolar cells. The lateral to medial gradient in CB1R expression during the first and second postnatal weeks is also opposite to the expression of functional glycine receptors present in the LSO of three week old gerbils [13]. If the gradient in glycine receptor expression reflects a gradient in MNTB synapse density, then one can speculate that cannabinoid signaling has contributed to the developmental elimination of glycinergic MNTB inputs to the LSO. In the adult rat, the tonotopic gradient of CB1R expression within

the LSO is absent, which is consistent with the idea that cannabinoid signaling is involved in the developmental adjustment of synaptic strength after hearing onset.

Future electrophysiological studies will be necessary to corroborate our anatomical results and to define the physiological conditions during which cannabinoid signaling is engaged in the LSO. This information will be useful to determine whether cannabinoid signaling at the MNTB-LSO synapses can induce LTD and whether cannabinoid signaling may mediate the topographic sharpening of functional MNTB-LSO maps that occur during the first postnatal week.[10, 18].

In most systems, endocannabinoid-mediated LTD is expressed presynaptically and involves a decrease in the probability of neurotransmitter release [1, 26, 28]. The anatomical co-localization of CB1R and VGLUT3 in MNTB neurons and their synaptic endings in the LSO suggests that CBR1R are expressed presynaptically at MNTB-LSO synapses.

In summary, results presented in this study provide anatomical evidence for cannabinoid signaling at developing GABA/glycinergic MNTB-LSO synapses. The presence of CB1R suggests that endocannabinoids may be a potential mechanism for synaptic silencing of MNTB-LSO synapses that underlies the topographic refinement of this auditory pathway before hearing onset.

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Highlights

- CB1R is present in the developing LSO and MNTB.
- Within the LSO, CB1R expression is higher in the lateral limb during the period before and around hearing onset.
- VGLUT3 and CB1R are highly colocalized in the LSO, indicating that the CB1R is present in the pre-synaptic MNTB terminals.

Figure 1.

Low and high magnification of coronal sections through the LSO and MNTB of a P5 (1A,B), P12 (1C,D), and adult (1E,F) rat. A. Overview of the anatomic structures. MNTB: medial nucleus of trapezoid body. SPON: superior periolivary nucleus. LSO. Lateral superior olive. 7n: facial nerve. B,D,E. High power magnification of corresponding sections of A,C, E. Individual axonal projections (arrow) to the LSO. Boutons demarcated with arrowheads. Scale bar: 100 μ m (A,C,E); 10 μ m (B,D,F)

Figure 2.

Relative grey values of the regions of the LSO. Three separate regions of interest (ROI) were analyzed in the medial (M), central (C), and lateral (L) limbs of the LSO. The relative grey values were calculated from each ROI, measuring 100 μm x 100 μm . The background (Bg) was determined as an area of neuropil dorsal to the LSO within the same section. The difference in the relative grey values was significant between the lateral vs. medial ($p=0.010$ at P5, $p=0.005$ at P12) limbs of the LSO in the P5 and P12 rats but not in the adult rat. Error bars represent SEM. Scale bar is 100 μm .

Figure 3.

Coronal section through the P5 rat LSO with double immunofluorescent labeling of VGLUT3 (red) and CB1R (green) [A-F]. D, E, F are enlarged images from inset square. VGLUT3 is a marker for MNTB terminals. Colabeling (yellow) is present in majority of the fibers. This finding demonstrates that CB1R is present in the presynaptic terminals within the LSO. Coronal sections through P5 MNTB (G-I) illustrates the colabeling of cell bodies of CB1 and VGLUT3 but axons labeled only for CB1 (green arrows). Scale bars for A,B,C: 100 μ m; D,E,F: 10 μ m; G,H,I: 25 μ m.