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Serotonin-immunoreactive neurons in the postnatal MAO-A KO mouse lateral superior olive project to the inferior colliculus

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ABSTRACT

During development, serotonin (5-HT) accumulates in thalamic, noradrenergic, and auditory brainstem neurons that are non-serotonergic in the adult. As demonstrated in somatosensory thalamocortical projections, this accumulation of 5-HT is necessary for the precise organization of afferent terminal arborizations. Accumulation of 5-HT in the auditory brainstem appears to be most robust in the lateral superior olive (LSO) and as demonstrated in the MAO-A knockout mouse, is present at birth and begins to taper off at postnatal day 7 (P7). During the same developmental period, 5-HT-positive terminal endings in the inferior colliculus (IC) have been reported to be more numerous than in the adult [O. Cases, C. Lebrand, B. Giros, T. Vitalis, E. De Maeyer, M. Caron, D. Price, P. Gaspar, I. Seif, Plasma membrane transporters of serotonin, dopamine and norepinephrine mediate serotonin accumulation in atypical locations in the developing brain of monoamine oxidase A knock-outs, J. Neurosci. 18 (1998) 6914–6927]. It has been hypothesized that the serotonergic terminal fibers in the IC belong to neurons whose cell bodies reside in the LSO. Here, we provide evidence based on morphological and tract-tracing data that LSO neurons containing serotonin in the perinatal mouse, project to the IC. These data suggest that, similar to thalamocortical projections in other sensory systems, 5-HT may play a role in regulating development of LSO terminal arbors in the IC.

In addition to its role as a neurotransmitter/neuromodulator in the adult central nervous system (CNS), increasing evidence supports a role for 5-HT in CNS development. During the perinatal period in rodents, 5-HT influences the development of sensory pathways. Changing 5-HT levels in the neonate alters the morphology of somatosensory and visual afferents at their targets [8,17,19,34]. For example, decreasing 5-HT levels reduces the terminal field size of somatosensory thalamocortical neurons [1,22], while increasing 5-HT levels disrupts the organization of thalamocortical terminal fields in somatosensory cortex [16] and impairs the segregation of eye-specific retinal terminal fields [33]. More recently, it has been shown that 5-HT depletion blurs the pattern of retinal afferents in the superior colliculus [9]. Such alterations have functional consequences as well. For example, stimulation-induced activation of the somatosensory pathway is significantly reduced in adult mice lacking the 5-HT transporter (5-HTT) gene compared to controls [6]. This effect was not observed in mice with the same genotype but in mice whose brain 5-HT levels were reduced, pharmacologically, during the neonatal period of development.

Few studies have investigated the developmental role of 5-HT in the central auditory system. Central auditory pathways are highly organized to maintain tonotopicity, to maintain the timing of neural cues necessary for sound localization, and to process spectral cues in the analysis of complex acoustic signals [35]. Given its highly organized projections, the auditory system may be useful as a model to investigate the role of 5-HT in the development of sensory projections throughout the brain. In particular, the projection from the lateral superior olive (LSO) to the inferior colliculus (IC) has been hypothesized to be developmentally influenced by 5-HT [3]. It is well known that LSO afferents are a major source of excitatory and inhibitory inputs to the central nucleus of the IC (CNIC; reviewed by [5,10,25]). Joining those from other brainstem regions, LSO axons enter the CNIC in the lateral lemniscus (LL); these fibers terminate in the CNIC forming a projection field of ventrolaterally to dorsomedially oriented rows (reviewed by [21]). These afferent inputs contribute to the fibrodendritic laminae, the primary morphological feature of the CNIC. Fibrodendritic laminae have been subdivided further into synaptic domains that form functional modules that likely form the substrate for temporal and spectral processing in the CNIC. LSO afferents in the CNIC exhibit an adult-like laminar appearance at birth, but continue to be refined during postnatal development, even before connections are made

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with the cochlea [2,7]. The factor(s) influencing refinement of the synaptic domains are not fully understood.

The somatosensory and visual system neurons whose development is known to be influenced by 5-HT levels are themselves non-serotonergic and utilize other neurotransmitters to communicate. However, during a short period of time which coincides with map refinement, these sensory neurons are said to "borrow" 5-HT and exhibit many features of a classical serotonergic neuron. For example, besides containing 5-HT, the neurons express the 5-HTT and the vesicular monoamine transporter 2 (see reviews of [8,19]). The neurons do not synthesize 5-HT, but acquire it from the extracellular space via the 5-HTT. Similarly, LSO neurons express features of the serotonergic phenotype during the postnatal period. In the postnatal mouse, cell bodies in the LSO are immunoreactive (IR) for 5-HT [3,28,29] and also express the 5-HTT and 5-HTT mRNA [3,31]. However, as with the other sensory systems, LSO neurons are not capable of synthesizing 5-HT, but take it up via the 5-HTT [32].

Thus, it appears that LSO neurons are similar to other non-serotonergic neurons that take up 5-HT and use it during development. The question remains as to the projection(s) of these particular LSO neurons. Do LSO neurons that accumulate 5-HT during development project to the IC? As one nucleus forming the superior olivary complex, the LSO is a heterogeneous nucleus comprised of five different neuronal types [10,26]. Of these, two major types of projection neurons have been identified—principal neurons that project to the IC and efferent neurons that project to the cochlea. Anecdotal evidence to date suggests that the LSO neurons that accumulate 5-HT during development are of the principal type that project to the IC. For example, it has been reported that the transient 5-HT-immunoreactivity in cell bodies of LSO neurons, postnatally, coincides with profuse numbers of 5-HT-IR axon terminals in the IC that decrease in the adult [3]. One could argue that these axon terminals belong to neurons of the serotonergic system (e.g. raphe neurons) which are known to innervate the IC of the adult [15]. However, these 5-HT-IR terminal endings observed in the CNIC (the specific IC target of LSO afferents), postnatally, are pericellular in arrangement. They do not resemble the afferents of true serotonergic neurons in the CNIC which terminate as *en passant* type fibers in the adult [13,29]. Also coincident with 5-HT-IR LSO cell bodies, axons in the LL (which has not been shown to carry axons of serotonergic system neurons) are transiently 5-HT-IR in the postnatal mouse [29]. These observations support the hypothesis originally proposed by Cases et al. [3] that 5-HT is taken up by LSO neurons that project to the IC. However, it has not been directly shown that these particular neurons project to the IC—that is the purpose of the current study. Neuroanatomical tract-tracing in conjunction with 5-HT immunohistochemistry was utilized to determine if postnatal LSO neurons that transiently take up 5-HT, actually do project to the IC.

Two different tract-tracers (wheat germ agglutinin-apohorseradish peroxidase-Au [WGAapoHRP-Au] and FluoroGold [FG]) were used to retrogradely label LSO neurons after injection into the IC; after cutting and processing to visualize retrogradely labeled cells, the sections were also processed, immunohistochemically, to simultaneously visualize 5-HT. To maximize detection of 5-HT in LSO neurons, monoamine oxidase A (MAO-A) knockout mice (which exhibit elevated 5-HT levels) were used. The mice were produced from homozygous breeding of MAO-A knockout mice from the original Tg8 strain (C3H/HeJ background; [4]). The mice were housed under standard humidity and temperature conditions with 12-h light/dark cycles. The day of birth (as documented at 8:00–8:30 AM) was designated as postnatal day 0 (P0). All procedures were performed in accordance with a protocol approved by the local Institutional Animal Care and Use Committee.

For the WGAapoHRP-Au injections, mice ($n = 3$ at P5) were anesthetized by hypothermia (placing on a piece of gauze sitting on top of crushed ice). A slit was made in the thin bone overlying the IC and a glass micropipette glued to a Hamilton syringe containing one of the retrograde tracers was lowered into the IC. Exactly 0.5 μl WGAapoHRP-Au (0.03 μg/μl in distilled water, E-Y Laboratories Inc.) was slowly (over a 5-min time period) pressure-injected into each IC. After recovery from anesthesia, the pups were returned to the litter. The following day, the pups were anesthetized with choral hydrate (5 mg/gm, i.p.) and perfused intracardially with phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in acetate buffer (pH 6.5), and then 4% paraformaldehyde with 0.05% glutaraldehyde in borate buffer (pH 9.5). The brains were removed and post-fixed for 2–4 days in the last fixative and then were rinsed in PBS for 1–2 days. The brains were equilibrated in 20% sucrose and then frozen sections (30–33 μm thick) cut in the coronal plane on a sliding microtome and collected in ice-cold PBS.

Immediately after cutting, the brain sections were processed to label the WGAapoHRP-Au. The WGAapoHRP-Au was visualized with the silver intensification technique using the IntenSE BL Silver Enhancement Kit (Amersham Life Sciences) as described previously (Thompson et al., 1995). After final rinses, the sections from one series were processed for 5-HT immunohistochemistry by our routine methods [27]. Briefly, after blocking non-specific binding, the sections were incubated in goat anti-5-HT conjugated to bovine serum albumin (BSA, ImmunoStar, 1:100,000) for 1–3 nights. Staining is completely eliminated by pretreatment of the diluted antibody with 100 μg of 5-HT/BSA conjugate. The antibody was labeled with the ABC method [12] using diaminobenzidine dihydrochloride (DAB) as the chromogen. After processing, the sections were mounted onto gelled slides, dried, and coverslipped with DPX (BDH Chemicals) for light microscopic analysis. Some sections were mounted onto gelled slides and coverslipped, after counterstaining with Neutral Red, to delineate the LSO. Neurons were considered double-labeled if they contained silver particles and brown DAB reaction product. For Fig. 1, photomicrographs of each panel were digitized (Olympus, DP 12 camera) under a 100× oil immersion objective at different focal planes (7 for Fig. 1A, 6 for Fig. 1B and 5 for Fig. 1D) and saved as jpegs at 72 dpi. The image in Fig. 1C was taken at a single focal plane. For Fig. 1A, B and D, each image was placed in a different layer (Corel PHOTO-PAINT 12). A base layer was selected (the layer containing the cell with the most obvious label) and the remaining layers were set at the "darken" setting at 100% opacity. Then each layer was sequentially merged with the base layer under the "visible" setting. The images were cropped and sized to 4 × 3 in. at 300 dpi and saved in tiff format. Slight color adjustments were made with the photo filter (deep blue at a density setting of 50% with luminosity preserved) and images sharpened. The images were imported into Corel Designer to assemble the figure with the accompanying magnification information.

In four other mice, FluoroGold (1 μl of 4% in distilled water, Fluorochrome, LLC) was injected into the IC in a similar manner bilaterally on P6. The following day, the mice were anesthetized and perfused as described above. The brains were removed and equilibrated in 20% sucrose in PBS. Frozen sections were cut along the transverse plane at 33 μm thick and collected in PBS with 0.1% NaN_3 for 5-HT immunohistochemistry with a fluorescent tag. The sections were rinsed in PBS-TX for 4 × 5 min each and then blocked in 10% normal rabbit serum and 1% BSA in PBS-TX for 2 h at room temperature. The sections were then incubated in anti-5-HT (made in goat, 1:500 in PBS-TX + 0.1% NaN_3) for 3 nights at 4 °C. The sections were then rinsed in Tris-buffered saline (TBS)-TX for 3 × 10 min each and then incubated in biotinylated horse anti-goat (1:100 in TBS-TX, Vector Labs) for 3 h at room temperature. The sections were rinsed 3 × 10 min each in BBS (0.1 M $NaHCO_3$ + 0.15 M NaCl; pH 8.5) and then placed in BBS containing Texas Red Avidin (1:500, Vector Labs) for 2 h at room temperature. The sections were rinsed in TBS for 3 × 5 min each, rinsed in 0.1 M PB, and then mounted