Glycoconjugate expression in the olfactory bulb of the premetamorphic larva of the Japanese sword-tailed newt (Cynops ensicauda)

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NOTE

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ABSTRACT. We examined the organization of the olfactory organ and assessed the lectin histochemistry to investigate the glycoconjugate distribution of the olfactory bulb in premetamorphic larvae of Cynops ensicauda. The nasal cavity was an oval chamber that contained olfactory epithelium and a primitive vomeronasal organ. Secretory products were found in the supporting cells of the two sensory epithelia and in the respiratory cells. Ten lectins bound to the olfactory and vomeronasal nerve fibers as well as to the glomeruli in the olfactory bulb. The binding intensity in larvae was weaker than that reported previously in mature animals. This difference suggests a functional correlation between the expression change of glycoconjugates and the developmental refinement of the olfactory system during metamorphosis.

KEY WORDS: lectin, metamorphosis, olfactory system, salamander, vomeronasal organ

Salamanders inhabit a wide variety of habitats, from aquatic to terrestrial environments. Most salamander species undergo a transition from an aquatic larva to a more terrestrial juvenile form, and often cross the threshold between aquatic and terrestrial habitats in the adult form. Metamorphosis during ontogenesis provides amphibians, including salamanders, a competitive advantage by reducing the competition for food sources between the larvae and adults that feed in two distinct habitats. Olfaction is a chemosensory system to detect chemical molecules emanating from a distant source, such as food items and individuals of the same or different species [2, 13]. During metamorphosis, the olfactory system in salamanders must adapt to a perception of water-soluble and/or volatile odorant molecules.

The olfactory system of amphibians is divided into two subsystems: the main and accessory (vomeronasal) olfactory systems [2, 13, 19]. In the main olfactory system, the olfactory epithelium (OE) in the nasal cavity contains three types of cells: receptor cells, supporting cells, and basal cells as a progenitor of the above two cells. Axons of the receptor cells form the olfactory nerve that projects to the main olfactory bulb (MOB) in the brain. The accessory olfactory system comprises the sensory epithelium in the vomeronasal (Jacobson’s) organ (VNE), the vomeronasal nerve, and the accessory olfactory bulb (AOB). The vomeronasal organ in amphibians is a diverticulum that branches off the main nasal cavity and its epithelium generally resembles the OE. Receptor cells in the VNE extend axons through the vomeronasal nerve to the AOB. The main olfactory system detects general odorants, while the accessory olfactory system detects unique stimuli, including some pheromones [6, 7, 13]. It is interpreted that the vomeronasal organ appeared in a common amphibian ancestor by the partitioning of a neuronal subpopulation of the OE that expressed vomeronasal-specific genes as a distinct organ [1, 13, 19]. In salamanders, the developmental variance of the accessory olfactory system seems to be complicated by both habitat diversity and metamorphosis. Schmidt et al. (1988) examined the primary olfactory and vomeronasal projections in ten salamander species with and without a habitat transition (the biphasic and direct-developing species, respectively) [15]. Vomeronasal projections of the biphasic species exhibit higher complexity, with termination fields in the AOB, than those of the direct-developing species, whereas the olfactory projections are similar in both types of species. In addition, an aquatic salamander with paedomorphosis, the mudpuppy (Necturus maculosus), lacks the vomeronasal organ, but another neotenic salamander, the axolotl (Ambystoma mexicanum), has an anatomically independent vomeronasal organ [1, 13, 19]. Previous studies have, therefore, focused on morphological comparisons of the olfactory systems among salamanders, between larval and adult stages, and between habitat types [1, 13, 17, 18].

The Japanese sword-tailed newt, Cynops ensicauda, is a semi-aquatic salamander that occurs in woodland habitats and is endemic to the Ryukyu Islands of Japan. Cynops ensicauda is strictly aquatic in the larval stage, but progressively transitions to a more terrestrial habit in the juvenile and adult life stages through the metamorphosis. This change from aquatic to terrestrial
habitats affords the opportunity to investigate the relationship between the development of the vertebrate olfactory system and the transition of living environments [1, 13, 18]. We previously determined the glycoconjugate expression in the primary olfactory center of mature *C. ensicauda* using the lectin histochemistry [9]. Lectins have a capacity to bind to specific sugar residues, and the lectin histochemistry is useful to visualize the glycoconjugate distribution in the olfactory system [6, 12]. Diverse glycoconjugates are expressed in the primary olfactory projection of vertebrates, and play an important role in cell-cell recognition and in guiding axons to their appropriate target during ontogenic development of the olfactory system [6, 12]. We hypothesized that if glycoconjugates act on the development and refinement of the primary olfactory projection during metamorphosis in salamanders, lectin bindings to the olfactory bulb should differ between larval and metamorphosed adult life stages of salamanders. We, therefore, determined the glycoconjugate distribution of the olfactory bulb in premetamorphic larvae of *C. ensicauda*, using the lectin histochemistry.

All procedures were carried out as approved by the Animal Care and Use Committee of the National Defense Medical College. Tadpoles of *C. ensicauda* from our laboratory stock were housed at approximately 20°C and fed three times a week with live brine shrimp. Sixteen- to eighteen-week-old larvae (n=6; body length: 32–38 mm; body weight: 0.28–0.35 g) that were grouped into developmental stage 58 based on the body appearance [20], were used in this study. Animals were anesthetized with 0.1% solution of Ethyl 3-aminobenzoate methanesulfonate (MS-222; MP Biomedicals, Santa Ana, CA, U.S.A.) and sacrificed by decapitation. Skulls and brains were placed in Bouin’s solution without acetic acid overnight for fixation, and routinely embedded in paraffin. Sections of the olfactory organ and brain were cut transversely and horizontally, respectively, at 5 µm and subjected to the hematoxylin-eosin staining, periodic acid-Schiff (PAS) staining, and lectin histochemistry analyses.

Ten biotinylated lectins, which exhibited a binding reactivity in the primary olfactory center of adult *C. ensicauda* [9], were used in this study (Lectin screening kits I–III, Vector Laboratories, Burlingame, CA, U.S.A.). The applied concentration and sugar specificity of lectins is shown in Table 1. Lectin histochemistry was performed as described previously [8]. In summary, deparaffinized sections were blocked with 1% bovine serum albumin in phosphate-buffered saline and incubated overnight at 4°C with biotinylated lectins. The reactions were detected with the Vectastain ABC-Elite kit (Vector Laboratories) and diaminobenzidine solution.

### Table 1. Concentrations, sugar specificities, and binding intensities of the lectins used in the glycoconjugate expression study involving the olfactory bulb of larval *C. ensicauda*

| Lectins (Abbreviation) | Conc. (µg/ml) | Primary sugar specificity | MOB NL | MOB GL | AOB NL | AOB GL |
|------------------------|--------------|--------------------------|-------|-------|-------|-------|
| Wheat germ agglutinin (WGA) | 1.0          | GlcNAc                   | +     | +     | +     | +     |
| Succinylated wheat germ agglutinin (s-WGA) | 10.0         | GlcNAc                   | +     | +     | +     | +     |
| Lycopersicon esculentum lectin (LEL) | 0.5          | GlcNAc                   | +++   | +++   | ++    | ++    |
| Soybean agglutinin (SBA) | 10.0         | GalNAc                   | +     | +     | +     | ±     |
| Bandeiraea simplicifolia lectin-I (BSL-I) | 2.0          | Gal                      | ++    | +     | +     | ±     |
| Peanut agglutinin (PNA) | 10.0         | Gal                      | -     | ±     | -     | ±     |
| Pisum sativum agglutinin (PSA) | 5.0          | Man                      | +     | +     | +     | +     |
| Lens culinaris agglutinin (LCA) | 5.0          | Man                      | ±     | ±     | ±     | ±     |
| Phaseolus vulgaris agglutinin-E (PHA-E) | 5.0          | Complex structures       | +     | +     | +     | +     |
| Phaseolus vulgaris agglutinin-L (PHA-L) | 5.0          | Complex structures       | ±     | ±     | ±     | ±     |

The lectin binding intensity in the nerve and glomerular layers (NL and GL) of the main and accessory olfactory bulb (MOB and AOB) was evaluated on a five-grade scale: -, negative; ±, faint; +, weak; ++, moderate; +++, strong. Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Man, mannose.
The olfactory bulb in larval *C. ensicauda* was found to be similar to that in adult animals [9]. The MOB occupied the rostralateral region of the telencephalon, and olfactory nerve fibers covered the surface of the olfactory bulb (Fig. 2a). The AOB was positioned caudally to the MOB, and the vomeronasal nerve coursed laterally to the olfactory nerve. The olfactory glomeruli of the MOB and AOB were distributed broadly and directly beneath the nerve layer (NL) that comprised of the olfactory and vomeronasal nerve fibers. All ten lectins showed a binding reactivity to the NL and glomerular layer (GL) in the olfactory bulb (Table 1). We scored the binding intensity of lectins in the olfactory bulb on a five-grade scale: -: negative, ±: faint, +: weak, ++: moderate, +++: strong. *Lycopersicon esculentum* lectin (LEL) and soybean agglutinin (SBA) showed a different binding in the NL and the GL, when compared between the MOB and AOB. LEL showed strong labeling in the NL and GL of the MOB, but bound moderately to the both of the AOB (Fig. 2b). SBA bound weakly to the NL of the MOB and AOB, and also distinguished the GL between the MOB and AOB by a different binding intensity: weak in the MOB, and faint in the AOB (Fig. 2c).

*Bandeiraea simplicifolia* lectin-I (BSL-I) and peanut agglutinin (PNA) showed a preferential reactivity to the NL and the GL, respectively, despite these having a similar reactivity between the MOB and AOB. The olfactory and vomeronasal NL were moderately labeled by BSL-I and the GL of the MOB and AOB were weakly labeled (Fig. 2d). PNA faintly labeled the GL of the MOB and AOB, but showed no labeling in the NL (Fig. 2e). The remaining six lectins [wheat germ agglutinin (WGA), succinylated wheat germ agglutinin (s-WGA), *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* agglutinin-E (PHA-E), and *Phaseolus vulgaris* agglutinin-L (PHA-L)] exhibited a uniform binding reactivity in the NL and GL of the olfactory bulb. The two layers were labeled with weak intensity by WGA (Fig. 2f), s-WGA (Fig. 2g), PSA (Fig. 2h) and PHA-E, and with faint intensity by LCA and PHA-L.

Adult *Cynops* possess the olfactory organ in a wide dorsoventrally flattened nasal cavity, and a vomeronasal organ that arises laterally from the main cavity as a diverticulum [10, 14]. In contrast, the nasal cavity of *C. ensicauda* larvae was oval with a shallow groove lined with the VNE. Previous studies have shown that neotenes and larvae of several salamanders exhibit an immature lateral nasal groove that contains the vomeronasal organ, in contrast to metamorphosed adults [16, 17]. These findings suggest that endocrine and metabolic modifications during metamorphosis would induce the development and maturation of the vomeronasal organ of larvae in some salamander species including *C. ensicauda*. Larval *C. ensicauda* showed a differentiated...
Fig. 2. Hematoxylin-eosin staining and lectin bindings in the main and accessory olfactory bulb (MOB and AOB) of larval *Cynops ensicauda*. The left and top sides of each panel indicate the rostral and lateral portions in hemispheres of the telencephalon, respectively. (a) Horizontal section of the olfactory bulb, stained with hematoxylin-eosin. The olfactory and vomeronasal nerves (ON and VN) terminated in the glomeruli of the MOB (MOB-GL) and in those of the AOB (AOB-GL), respectively. (b–h) LEL (b) and SBA (c) exhibited a different binding intensity between the MOB and AOB. BSL-I (d) and PNA (e) showed a different preference for binding between the nerve and glomerular layers. WGA (f), s-WGA (g), and PSA (h) bound with a uniform labeling to the ON and VN and to the MOB-GL and AOB-GL. Arrows in the panels represent the border between the MOB and AOB. Scale bar: 100 µm.
arrangement of cells in the OE and VNE, but lacked PAS-positive Bowman’s gland in the lamina propria. In most tetrapods, respiratory and supporting cells and Bowman’s glands secrete specialized products onto the surface of the olfactory sensory epithelium [2, 5]. These secretory products contain various compounds, including odorant binding proteins, and play a role in sensing odorant molecules. In general, Bowman’s glands are absent in fish and some aquatic amphibians [5, 13]. We found PAS reactions in supporting cells of the OE and VNE and in respiratory cells of non-sensory ridges in C. ensicauda larvae. It appeared that PAS-positive products may have been secreted from the two cell types onto the free border of the sensory epithelium, where the PAS reaction was also observed. Our preliminary investigations showed that receptor cells in the OE of larval C. ensicauda expressed the subunit Gαolf of G-protein (unpublished data), which is an essential factor in the olfactory signal transduction [6, 10], suggesting a functional maturity of receptor cells. The olfactory reception in aquatic premetamorphic salamanders may, therefore, be sustained by secretory products of the respiratory and supporting cells.

Our results demonstrated that the glycoconjugate expression characterized the nerve fibers and olfactory glomeruli in both the MOB and AOB of premetamorphic C. ensicauda. Of the ten lectins used in the present study, LEL and BSL-I showed more preferential binding to the primary projections in the olfactory bulb than the others. LEL and BSL-I have an affinity for N-acetylglucosamine and galactose, respectively, and these glycoconjugate expressions in larval C. ensicauda may play a role in the axonal growth and fasciculation of the primary olfactory projection, as previously seen in rodents [12]. Regarding the comparison of lectin binding to the olfactory bulb among mature salamanders, a species-specific glycoconjugate expression was demonstrated by some lectins [3, 4, 8, 9]. In the present study, the ten lectins (see Table 1) showed a bidding reactivity to the olfactory bulb of larval C. ensicauda. Labeling of these lectins also showed positive results with the olfactory bulb of the adults [9], and it suggests that the glycoconjugate types expressed in the primary olfactory projection are well conserved from the premetamorphic larval stage to the mature stage. Interestingly, in larvae of C. ensicauda, the lectin reactivity tended to exhibit a weaker binding reaction in both the MOB and AOB than that in the adult [9]. The developmental change of lectin bindings in the olfactory system has also been described in rodents [6, 11, 12]. In mouse (Mus musculus), Dolichos biflorus agglutinin (DBA) first labels axon bundles of the olfactory nerve at embryonic stage day 16, and subsequently labels the olfactory glomeruli of the MOB at the prenatal stages [11]. At the postnatal developmental stage, DBA binding is initiated in the vomeronasal nerve and the glomeruli of the AOB. This indicates a developmental change of the glycoconjugate expression in the primary olfactory center, and suggests that glycoconjugates in the primary projection may be involved in the maturation of axonal fasciculations and neuronal connections [12]. Our studies have revealed that there is a difference in the density of glycoconjugates in the olfactory bulb between premetamorphic and adult C. ensicauda, and suggest that there is a common correlation between the developmental change of lectin bindings and the maturation of the olfactory system among vertebrates. Interestingly, larval C. ensicauda did not represent a rostral subset in olfactory glomeruli of the MOB that was defined in adults by a dense expression of glycoconjugates [9]. Larval salamanders are fully aquatic and sense only water-soluble odorant molecules, so the primary projection to the rostral MOB may be immature in this stage of development. This seems to result in the homogenous glycoconjugate expression among glomeruli in the MOB of larval C. ensicauda. Further analysis of the anatomical comparison throughout various ontogenetic stages of salamanders may provide greater insights into the development of the olfactory system in relation to habitat transition in metamorphic animals.

CONFLICT OF INTERESTS. None of the authors have any conflicts of interest associated with this study.

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