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Differential Localization of G-proteins G_i and G_o in the Accessory Olfactory Bulb of the Rat

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To clarify the functional differences among G-proteins, we investigated the localization of G_i and G_o in the olfactory bulb of rats by both immunohistochemical and immunochemical techniques, using purified antibodies specific to the α-subunits of G_{i_1} ($G_{i_1}\alpha$), G_{i_2} ($G_{i_2}\alpha$), and G_o ($G_o\alpha$), respectively. We found that $G_{i_2}\alpha$ is localized exclusively in the accessory olfactory bulb, but it is present at only low levels in the main olfactory bulb. The unique pattern of immunoreactivity specific for $G_{i_2}\alpha$ and $G_o\alpha$ within the glomeruli of the accessory olfactory bulb and the results of immunoassays indicate that the accessory olfactory bulb is divided into two parts: the anterior region is rich in G_{i_2}, while the posterior region is rich in G_o. These findings suggest that the accessory olfactory bulb has two different functions. In addition, we found that the concentration of $G_{i_2}\alpha$ in the accessory olfactory bulb increases during puberty and reaches the adult level at 12 weeks after birth, while that in the main olfactory bulb remains constant. By contrast, the concentrations of $G_o\alpha$ in the accessory olfactory bulb and the main olfactory bulb increase with similar kinetics. These findings suggest that G_{i_2} is a key protein in signal transduction in the accessory olfactory bulb, and increases in its level seem to be related to sexual maturation.

There are two olfactory pathways in vertebrates, the main and the accessory pathways. The main olfactory pathway, which originates in the sensory epithelium in the nasal cavity and ends in the main olfactory bulb, is involved in the perception of volatile olfactory stimuli. By contrast, the receptor neurons in the vomeronasal organ, which consists of bilaterally paired tubes lying in the ventral portion of the nasal cavity, project to the accessory olfactory bulb through the vomeronasal nerves (McCotter, 1912), and this pathway seems to be involved in the olfaction of nonvolatiles (Wysocki et al., 1980). Destruction of the accessory olfactory pathway impairs the reproductive functions of experimental animals (Powers and Winans, 1971; Meredith, 1986; Saito and Moltz, 1986), suggesting that the accessory olfactory system plays an important role in the perception of intersex smell.

G-proteins are a family of signal-coupling proteins, and each is a heterotrimer composed of α-, β-, and γ-subunits. In the sensory organs of vertebrates, such as the olfactory epithelium and the retina, tissue-specific G-proteins function as active signal transducers. Certain odorants stimulate adenylyl cyclase through G_{olf} (Jones and Reed, 1989) and G_s (Mania-Farnell and Farbman, 1990), and transducin regulates the activity of cGMP phosphodiesterase in response to activation by light of the photoreceptor rhodopsin (Gilman, 1987). There are also many reports suggesting that G_i and G_o are coupled with several receptors and regulate adenylyl cyclase activity, Ca^{2+} and K^+ channels, and phospholipase C activity (Kikuchi et al., 1986; Hescheler et al., 1987; Neer and Clapham, 1988; VanDongen et al., 1988; Moriarty et al., 1990). However, it is still unclear whether the three subtypes of G_i (G_{i_1}, G_{i_2}, and G_{i_3}) and G_o have individual and specific functions. G_o and G_{i_1} are mainly localized in the neuropil of the brain. G_{i_2} is widely distributed in various tissues, including the brain, but concentrations of G_{i_2} in the brain are much lower than those of G_{i_1} and G_o. To clarify the functional differences between G_i and G_o, we investigated the localization of these G-proteins by use of purified antibodies specific to the respective α-subunits of G_{i_1} ($G_{i_1}\alpha$), G_{i_2} ($G_{i_2}\alpha$), and G_o ($G_o\alpha$) in the rat olfactory bulb. We report here that $G_{i_2}\alpha$ is expressed at an extremely high level in the accessory olfactory pathway and that its concentration increases with postnatal development, probably in close association with sexual maturation. We also show that the accessory olfactory bulb is composed of two distinctive regions, one of which is rich in G_{i_2} while the other is rich in G_o.

Materials and Methods

Immunohistochemical study. Five male rats (12 weeks old) were deeply anesthetized with diethyl ether and perfused with a fixative solution (Schmechel et al., 1980) composed of 4% paraformaldehyde, 1% glutaraldehyde, 0.2% picric acid, and 2% sucrose in 0.1 M sodium acetate buffer, pH 6.0. This fixative has been shown to be suitable for immunohistochemical studies of the α-subunits of G_o (Asano et al., 1987, 1988; Semba et al., 1990), G_{i_1} (Asano et al., 1990), and G_{i_2} (Asano et al., 1989a). The dissected olfactory bulbs were kept in the same fixative for 4 hr. After rinsing with and left standing in 50 mM Tris-HCl buffer (pH 7.4) overnight, the tissues were dehydrated through a graded series of alcohols and embedded in paraffin. Serial sections (5 μm) of the main and the accessory olfactory bulbs from rats were prepared and immunostained by the indirect peroxidase-labeled antibody method (Nakane, 1975), using purified antibodies against $G_{i_1}\alpha$, $G_{i_2}\alpha$, $G_o\alpha$, and $\beta\gamma$. For controls, antibodies preabsorbed with the respective purified G-proteins were used, and these antibodies were not associated with any positive staining. The specificity of these antibodies has been described elsewhere (Asano et al., 1987, 1989a, 1990; Morishita et al., 1988). Production of the antibodies can be summarized as follows. $G_o\alpha$- and $\beta\gamma$-subunits were purified from bovine brain, while $G_{i_1}\alpha$ and $G_{i_2}\alpha$ were purified from bovine lung. The antisera were raised in rabbits by injection of the purified G-proteins, and the antibodies were purified from antisera by the use of appropriate antigen-coupled Sepharose columns. Antibodies against $G_o\alpha$ reacted only with $G_o\alpha$ and did not cross-react with $G_i\alpha$- or

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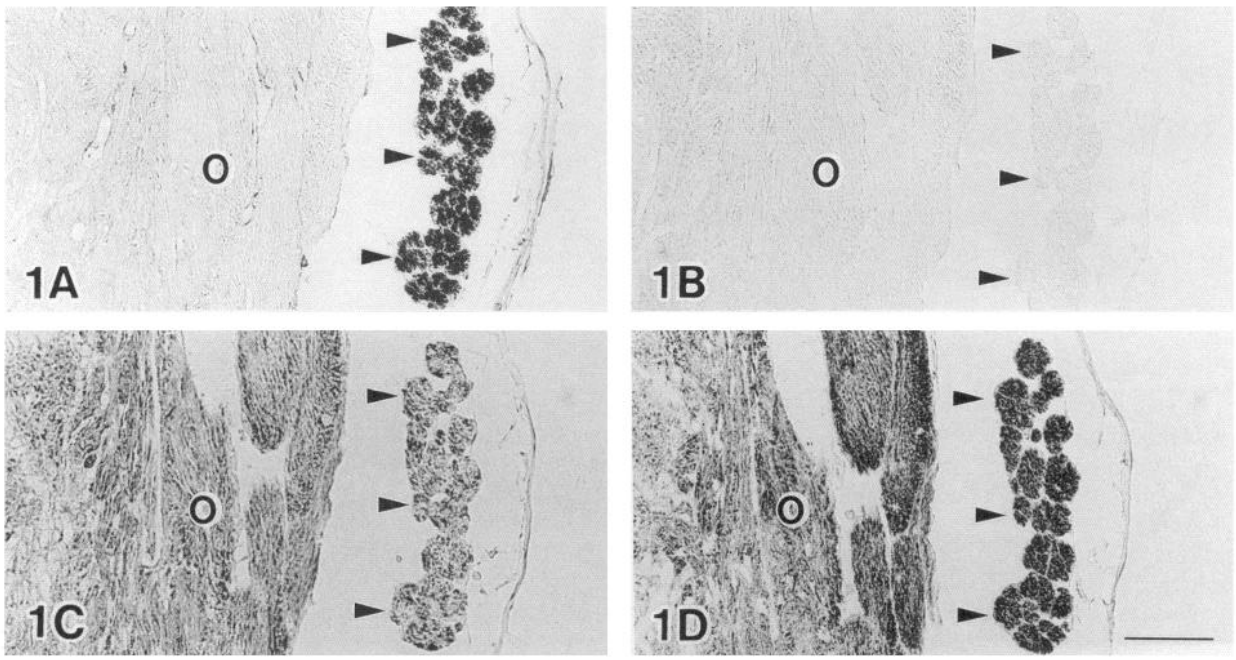

Figure 1. Serial sections of the main olfactory bulb and the vomeronasal nerves, immunostained with antibodies against $G_{i2}\alpha$ (*A*), $G_{i1}\alpha$ (*B*), $G_o\alpha$ (*C*), and $\beta\gamma$ (*D*). The vomeronasal nerves (*arrowheads*) between bilateral olfactory bulbs are intensely immunopositive for $G_{i2}\alpha$, $G_o\alpha$, and $\beta\gamma$ but almost immunonegative for $G_{i1}\alpha$. By contrast, the olfactory nerve layer (*O*) in the main olfactory bulb is immunopositive only for $G_o\alpha$ and $\beta\gamma$. Note that the vomeronasal nerves are homogeneously immunopositive for $\beta\gamma$, but they are not homogeneously immunopositive for $G_{i2}\alpha$ and $G_o\alpha$. Scale bar, 50 μm.

$\beta\gamma$-subunits in an immunoblot assay. Antibodies against $G_{i1}\alpha$ (Asano et al., 1990) cross-reacted with $G_{i3}\alpha$, but not with $G_{i2}\alpha$ or $G_o\alpha$. Because the concentration of G_{i3} seems to be very low in the brain (Goldsmith et al., 1988; Kanaho et al., 1989), the antibodies are referred to as anti-$G_{i1}\alpha$. Antibodies against $G_{i2}\alpha$ reacted only with $G_{i2}\alpha$, but not with $G_{i1}\alpha$, $G_{i3}\alpha$, or $G_o\alpha$. Antibodies against the $\beta\gamma$-subunits reacted mainly with the 36 kDa β-subunit. Since $\beta\gamma$-subunits are common to all G-proteins, antibodies against $\beta\gamma$ were also used to confirm the presence of the α-subunit of G_o and G_i.

Enzyme immunoassay procedures. Male rats of various ages were deeply anesthetized with diethyl ether, and the main and the accessory olfactory bulbs were dissected out under a stereomicroscope with ophthalmologist's scissors. The anterior and the posterior parts of the accessory olfactory bulb of five male rats (16 weeks old) were dissected out in the same way. The tissues were kept frozen at −80°C until analysis. The frozen tissues of the accessory olfactory bulb were homogenized at 0°C with a Potter–Elvehjem homogenizer in 100 μl of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM β-mercaptoethanol, and 1% sodium cholate. The main olfactory bulbs were homogenized in 9 vol (v/w) of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM β-mercaptoethanol, and 2% sodium cholate. The homogenate was sonicated for 20 sec and then centrifuged at 4°C at 100,000 × *g* for 1 hr. The supernatant fractions were used for the immunoassay of $G_{i2}\alpha$ (Asano et al., 1989a) and $G_o\alpha$ (Asano et al., 1987) after dilution. Less than 10% of total GTPγS-binding activity remained in the insoluble fraction when this procedure was followed (Asano et al., 1987). Proteins were quantitated by the method of Schaffner and Weissmann (1973).

Results

The vomeronasal nerves running between the right and the left main olfactory bulbs were intensely stained after immunoreactions specific for $G_{i2}\alpha$, $G_o\alpha$, and $\beta\gamma$, but they were almost immunonegative for $G_{i1}\alpha$. In addition, the vomeronasal nerves were homogeneously immunopositive for $\beta\gamma$, but they were not homogeneously immunopositive for $G_{i2}\alpha$ and $G_o\alpha$. This observation suggests that not all of the sensory neurons in the vomeronasal organ are $G_{i2}\alpha$ and $G_o\alpha$ positive (Fig. 1). By contrast, the olfactory nerve fibers were almost immunonegative for $G_{i2}\alpha$ and $G_{i1}\alpha$, but they were intensely immunoreactive for $G_o\alpha$ and $\beta\gamma$ (Fig. 1). In the accessory olfactory bulb, the pattern of staining of the glomeruli with the various antibodies revealed that the anterior region and the posterior region could be distinguished from one another. The glomeruli in the anterior region were intensely immunoreactive for $G_{i2}\alpha$ but only faintly so for $G_o\alpha$, while those in the posterior region were intensely immunopositive for $G_o\alpha$ but only faintly so for $G_{i2}\alpha$ (Fig. 2*A,C*). Although the glomeruli in the anterior and the posterior regions were weakly immunopositive for $G_{i1}\alpha$, the immunoreactivity was greater in the posterior region (Fig. 2*B*). The glomeruli in the anterior and the posterior regions were equally immunoreactive for $\beta\gamma$ (Fig. 2*D*). The molecular layer reacted to an equal extent with each of these antibodies (Fig. 2). To extend this finding, we quantitated the concentrations of $G_o\alpha$ and $G_{i2}\alpha$ in the anterior region and the posterior region of the accessory olfactory bulb by immunoassay. The results of the immunoassays revealed that the concentration of $G_{i2}\alpha$ was higher in the anterior region, while the concentration of $G_o\alpha$ was higher in the posterior region (Fig. 3).

Because the accessory olfactory system seems to participate in sexual behavior, we next examined whether the concentration of $G_{i2}\alpha$ in the accessory olfactory bulb increases during sexual maturation. We previously reported that the concentration of $G_{i2}\alpha$ in the rat cerebral cortex was almost constant throughout postnatal development (Asano et al., 1989a). In the main olfactory bulb, the concentration of $G_{i2}\alpha$ was also constant in rats of all ages examined (Fig. 4). By contrast, the concentration of $G_{i2}\alpha$ in the accessory olfactory bulb increased markedly at puberty and reached the adult level at 12 weeks after birth. The concentration of $G_{i2}\alpha$ in the accessory olfactory bulb of rats at 12 weeks after birth (37.4 ± 1.4 pmol/mg protein) was 2.6-fold

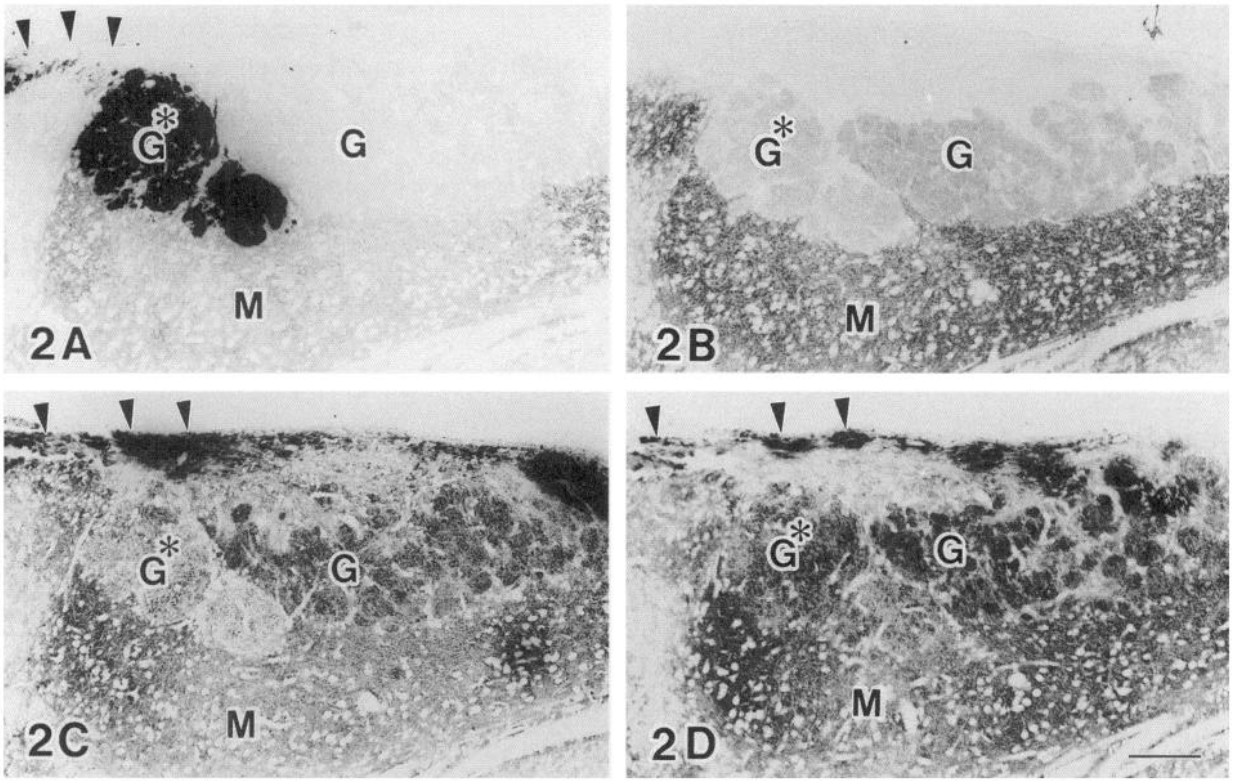

Figure 2. Serial sections of the accessory olfactory bulb and the vomeronasal nerves immunostained with antibodies against $G_{i2}\alpha$ (*A*), $G_{i1}\alpha$ (*B*), $G_o\alpha$ (*C*), and $\beta\gamma$ (*D*). The vomeronasal nerves are immunopositive for $G_{i2}\alpha$, $G_o\alpha$, and $\beta\gamma$ (*arrowheads*), but they are almost immunonegative for $G_{i1}\alpha$. The glomeruli are intensely immunopositive for $G_{i2}\alpha$ in the anterior region (*G**) but only faintly so in the posterior region (*G*). By contrast, the glomeruli are intensely immunopositive for $G_o\alpha$ in the posterior region (*G*) but only weakly so in the anterior region (*G**). The glomeruli are weakly immunopositive for $G_{i1}\alpha$, but the immunoreactivity is greater in the posterior region (*G*) than in the anterior region (*G**). All glomeruli in the accessory olfactory bulb are equally immunopositive for $\beta\gamma$. The molecular layers of the anterior and the posterior regions (*M*) are equally immunopositive for each antibody. Scale bar, 100 μm.

higher than that at 2 weeks after birth (14.6 ± 0.8 pmol/mg protein). The concentration of $G_o\alpha$ in both the main and the accessory olfactory bulbs increased during postnatal development. However, the rapid increase in concentration of $G_o\alpha$ in the main olfactory bulb occurred earlier than the increase in concentration of $G_{i2}\alpha$ in the accessory olfactory bulb; the concentration of $G_o\alpha$ in the main olfactory bulb of rats at 12 weeks after birth (59.4 ± 1.9 pmol/mg protein) was only 1.2-fold higher than that at 2 weeks after birth (47.7 ± 2.3 pmol/mg protein). The concentration of $G_o\alpha$ in the accessory olfactory bulb increased with similar kinetics to the increase in the concentration of $G_o\alpha$ in the main olfactory bulb, but it reached the adult level slightly later. In addition, there was no significant difference in the concentration of $G_{i2}\alpha$ between adult males and females (data not shown).

Discussion

The use of urine to mark the environment is a well-known behavioral trait of many vertebrates. The vomeronasal organ is involved in the detection of nonvolatile odorants in the urine (Wysocki et al., 1980), and major histocompatibility complex antigens are excreted in rat urine as olfactory recognition cues (Singh et al., 1987) for a wide range of phenotypic characteristics, such as species, strain, and sex of the subject (Beauchamp et al., 1985). In yeast, the mating pheromone response is activated by a G-protein–mediated signaling pathway in which $\beta\gamma$ is previously thought to be the active transducer of the signal in *Saccharomyces cerevisiae* (Whiteway et al., 1989). By contrast, it is reported recently that the α-subunit appears to function as a positive factor that transmits the signal from the mating-factor receptors to a downstream effector(s) in *Schizosaccharomyces pombe* (Obara et al., 1991).

In the mammalian brain, $G_{i2}\alpha$ appears to be a minor G-protein and it is distributed almost uniformly in the neuropil-rich regions (Asano et al., 1989a, 1990). By contrast, the concentration of $G_{i2}\alpha$ in the accessory olfactory bulb in the present study is the highest found by us in any tissues or cells examined (Asano et al., 1989a,b). Therefore, the present results suggest that $G_{i2}\alpha$ is one of the major G-proteins in the accessory olfactory bulb and plays a crucial role in signal transduction, as is the case for transducin (Gilman, 1987) in the retina and for G_{olf} (Jones and Reed, 1989) in the olfactory epithelium.

During the development of the cerebral cortex in rats, the concentration of $G_{i2}\alpha$ was constant but that of $G_o\alpha$ increased markedly and approached the adult level within 3 weeks after birth (Asano et al., 1988). The developmental changes in the concentrations of $G_{i2}\alpha$ and $G_o\alpha$ in the main olfactory bulb in the present study were similar to the results obtained in the cerebral cortex. We have also demonstrated a significant increase in the concentration of $G_o\alpha$, with little change in that of $G_{i2}\alpha$, in pheochromocytoma PC12 cells and in neuroblastoma × glioma hybrid NG108-15 cells that had been stimulated to

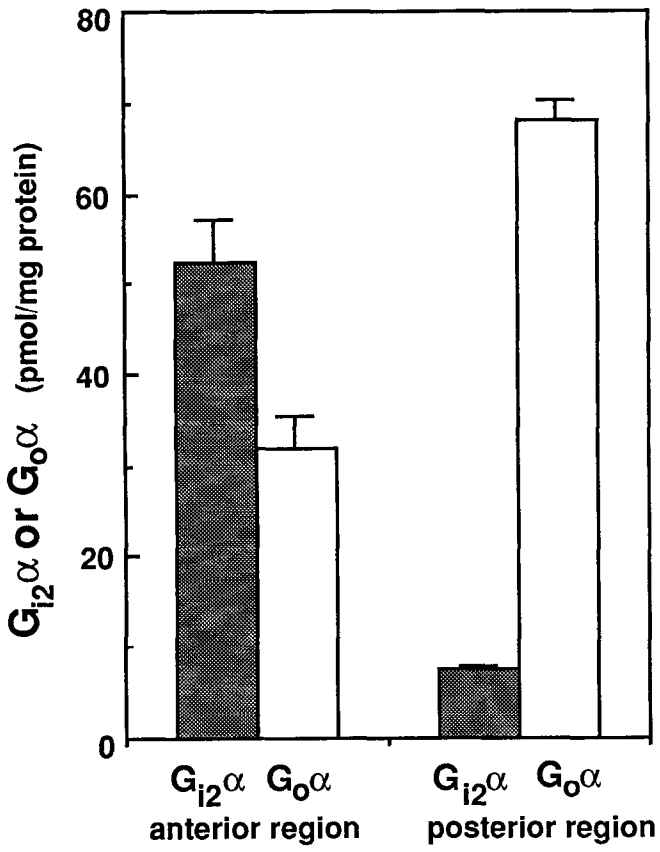

Figure 3. Immunoassay of $G_{i2}\alpha$ and $G_o\alpha$ in the two regions of the accessory olfactory bulb. The concentration of $G_{i2}\alpha$ in the anterior region is higher than that in the posterior region. By contrast, the concentration of $G_o\alpha$ in the posterior region is higher than that in the anterior region. Each column shows the mean ± SE of results from five samples.

differentiate by treatment with NGF or forskolin (Asano et al., 1989b). From these results, it appears that the concentration of $G_o\alpha$ increases during the maturation of nervous tissues while that of $G_{i2}\alpha$ does not. By contrast, the concentration of $G_{i2}\alpha$ in the accessory olfactory bulb increased during puberty and reached the adult level at 12 weeks after birth, lagging behind the maturation of nervous tissues. Therefore, our findings support the hypothesis that the gene for G_{i2} is expressed during sexual maturation in the accessory olfactory bulb and plays an important role in the perception of intersex smells, such as those associated with pheromones. It is also possible that the concentration of $G_o\alpha$ in the accessory olfactory bulb increases further as a result of sexual maturation.

The present results also indicate that the accessory olfactory bulb consists of two regions; the anterior region is rich in $G_{i2}\alpha$ and the posterior region is rich in $G_o\alpha$. It has been reported that carbohydrate antigens that are detectable with monoclonal antibodies are localized heterogeneously in the accessory olfactory bulb (Schwarting and Crandall, 1991) and in the vomeronasal nerve (Mori, 1987), as was observed in the case of G-proteins in the present study. It seems likely, therefore, that the vomeronasal-accessory pathway consists of at least two systems, one in which G_{i2} is involved and one in which G_o is involved.

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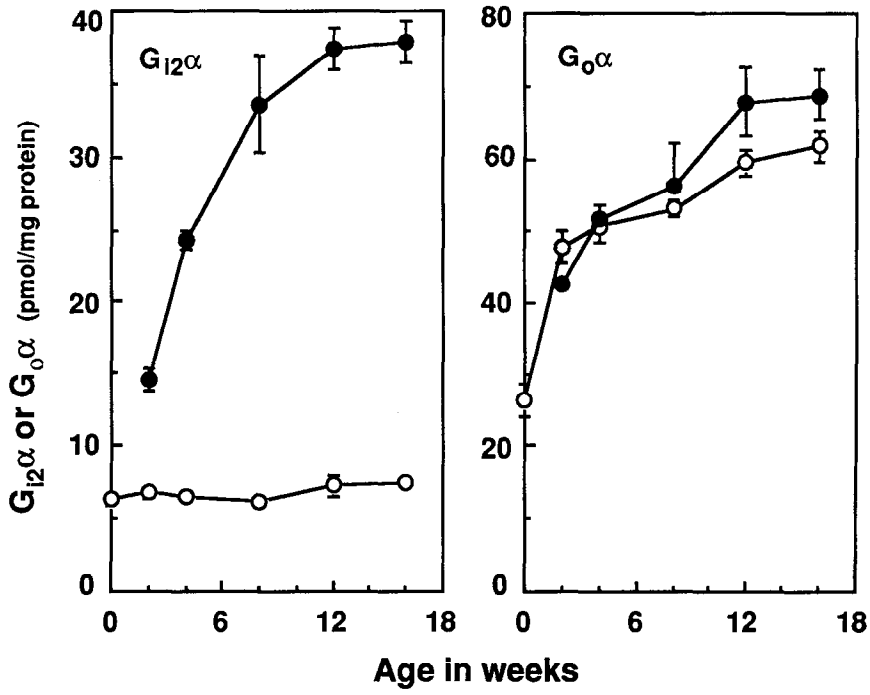

Figure 4. The changes in concentrations with development of $G_{i2}\alpha$ and $G_o\alpha$ in the accessory (*solid circles*) and the main olfactory bulb (*open circles*). The concentration of $G_{i2}\alpha$ in the accessory olfactory bulb increases at puberty and reaches the adult level 12 weeks after birth, while that in the main olfactory bulb remains relatively constant. Concentrations of $G_o\alpha$ in the accessory and the main olfactory bulb increased in parallel during postnatal development. Each point represents the mean ± SE of results from five animals.

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