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Distribution of G-protein α Subunits and Neurotransmitter Activation of $G_{\alpha i}$ and $G_{\alpha q}$ in the Brain of the Lobster *Homarus americanus*

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ABSTRACT

Immunocytochemistry using antisera specific for the G-protein α subunits $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ revealed similar patterns of immunoreactivity in the lobster brain. Immunoreactivity was strongest in neuropil, especially the olfactory and accessory lobes, and was characterized by bundles of fine threads leading to dense concentrations of punctate staining in the glomeruli. This may reflect the concentration of G-protein α subunits at synapses. The major differences between the antisera were distinct patterns of staining intensity in subregions of glomeruli of the olfactory and accessory lobes. This result is potentially correlated with previous evidence that these subregions are neurochemically distinct. Neuronal cell bodies contained moderate levels of immunoreactivity at the plasma membrane and faint staining in the cytoplasm. The olfactory globular tract was moderately immunoreactive, but other fiber tracts were weakly immunoreactive. Immunoreactivity in the deutocerebral commissure consisted of small oval cell bodies and strands that formed a reticulated pattern, suggestive of glia. Photoaffinity labelling by using an analog of GTP demonstrated that histamine activated $G_{\alpha i}$ in brain homogenates. Further evidence of G-protein activation was obtained by showing that stimulation with a mixture of neuroactive substances increased the amount of phospholipase C-β associated with membranes, $G_{\alpha q}$, and G_{β}. The lobster brain, especially in its neuropil regions, is richly endowed with neuromodulatory biochemical pathways involving $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$. J. Comp. Neurol. 422:402–414, 2000.

Indexing terms: GTP-binding proteins; crustacea; arthropoda; signal transduction; neurotransmission; olfaction

Decapod crustaceans have long been used to study neuronal processes such as complex rhythmic behavior of neural networks, the neurohormonal regulation of behavior, neural development, and the processing of sensory information (Wiese et al., 1990). A common theme in these studies has been the ability of neuroactive substances, primarily biogenic amines and peptides, to modulate and coordinate neural activity and behavior (Kravitz et al., 1984; Atwood et al., 1989; Weimann et al., 1993; Schmidt and Ache, 1994a; Blitz et al., 1995; Johnson et al., 1995; Zhang and Harris-Warrick, 1995; Yeh et al., 1996). The ability of the amines serotonin, octopamine, dopamine, and histamine to regulate neural activity and alter behavior was recently reviewed by Beltz (1999). A variety of peptides also modulate neural activity and behavior in crustaceans. A good example is found in the studies of the pyloric and gastric mill rhythms produced by the stomatogastric ganglion (Nusbaum and Marder, 1989; Golowasch and Marder, 1992; Weimann et al., 1993; Skiebe and Schneider, 1994; Turrigiano et al., 1994; Blitz et al., 1995). The locations of many neuroactive substances within the crustacean nervous system have been identified (Beltz and Kravitz, 1983; Siwicki and Bishop, 1986; Kobierski et al., 1987; Orona et al., 1990; Sandeman et al., 1992; Cour-

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nil et al., 1994; Schmidt and Ache, 1994a,b, 1997; Blitz et al., 1995; Christie et al., 1995a,b; Langworthy et al., 1997). Therefore, a wealth of information exists about the location of bioactive amines and peptides within the crustacean nervous system and about many of the physiologic roles of these substances.

However, there is a paucity of information about the molecular and biochemical mechanisms that underlie the modulatory actions of these substances in crustaceans. The identity of the neuroactive substances involved predicts that the predominant mechanisms will involve activation of G-protein-dependent pathways, but this has rarely been demonstrated. The few exceptions are studies of neuromuscular junctions, where adenylyl cyclase is activated by serotonin and octopamine, and where phospholipase C is activated by serotonin (Battelle and Kravitz, 1978; Atwood et al., 1989; Dixon and Atwood, 1989; Goy and Kravitz, 1989). We recently have isolated cDNA clones for several subunits of heterotrimeric G-proteins from the American lobster *Homarus americanus* (McClintock et al., 1992, 1997; Xu et al., 1997, 1998). These clones have provided tools for describing the distribution of these G-proteins within the lobster brain and to begin to investigate their activation by neuroactive substances. We have used these tools to detect the patterns of expression of $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ in the lobster brain, and to provide evidence that neurotransmitters can stimulate $G_{\alpha i}$ and $G_{\alpha q}$ in homogenates of lobster brain.

MATERIALS AND METHODS

Lobsters

American lobsters were purchased from Falmouth Fish Market, Falmouth, MA. Lobsters were held in artificial seawater at 4°C for no more than 2 weeks.

Immunocytochemistry

Brains were dissected from nine lobsters. Six of these brains provided adjacent sections that were stained with different antisera. Each of the three antisera were also reacted with all sections from a single brain. The brains were partially desheathed and fixed in 4% paraformaldehyde as described (Xu et al., 1998). Mounting in gelatin and sectioning were performed as described (Schmidt and Ache, 1997), except that the gelatin blocks were fixed in 4% paraformaldehyde before sectioning, which was done at room temperature. Vibratome sections were made at 25–35 μm thickness. Antisera C-10 ($G_{\alpha i}$), C-19 ($G_{\alpha q}$), and C-18 ($G_{\alpha s}$) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These antisera recognize C-terminal peptides whose sequences differ between the subtypes of G-protein α subunits. However, these sequences are conserved within each subtype of G-protein α subunit across many eukaryotic taxa, including lobsters (McClintock et al., 1992, 1997; Xu et al., 1997). The antisera were used at dilutions of 1:200 or 1:300, 1:200 or 1:300, and 1:150 or 1:200, respectively. For preabsorption controls, each antiserum was incubated for 1 hour with a 10-fold molar excess of its antigenic peptide. Immunocytochemistry was performed as described by Xu et al. (1998) by using a blocking solution of Sorensen's phosphate buffer, pH 7.4, containing 0.3% Triton X-100 and 10% normal goat serum. Wide-field fluorescence micrographs were obtained on slide film or direct digital acquisition by using a Nikon 300 inverted microscope equipped with a Nikon 6000 35-mm camera and a Kodak Megagrabber Plus digital camera. Confocal fluorescence images were obtained by using a Leica TCS Confocal System. Images from slides were digitized by scanning. Digital gray-scale images were adjusted to stretch pixel intensities from minimum to maximum. To compare intensity differences in staining between regions of columnar glomerular neuropils, the ratio of the intensity of the base region to the intensity of the cap/subcap region of single glomeruli was calculated from unadjusted digital images. NIH Image software was used to measure the average intensity of fluorescence. Descriptions of brain anatomy conform to the nomenclature proposed by Sandeman et al. (1992).

Photoaffinity labelling

We synthesized *m*-acetylanilido-GTP (AcA-GTP), a photoreactive analog of GTP, as described by Zor et al. (1995). To label $G_{\alpha i}$ in homogenates of lobster tissue, we modified a published method (McKenzie, 1992) by optimizing the activation buffer for lobster (in mM): 150 NaCl, 37.5 Tris, pH 7.4, 2.5 $MgCl_2$, 10 EGTA, 2.6 Ca^{2+} (to give free calcium at 10 nM), 1 ATP, and 0.25 % BSA. Brain homogenates were prepared in CT buffer (in mM: 120 NaCl, 5 KCl, 1.6 K_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 7.5 glucose, 2 EGTA, 3 μg/ml Pefabloc (Boehringer Mannheim, Indianapolis, IN), 1 μg/ml pepstatin, 1 μg/ml leupeptin, 10 μg/ml benzamidine, pH 7.4) and either stored as aliquots at −80°C, or used to prepare a membrane fraction according to a procedure previously described for eye-eyestalk homogenates (Xu and McClintock, 1999). Aliquots of these lobster brain membranes containing 10 μg of protein were also stored at −80°C. For photoaffinity labelling, membrane aliquots were thawed on ice and suspended at 28°C in activation buffer containing protease inhibitors (3 μg/ml Pefabloc, 1 μg/ml pepstatin, 10 μg/ml leupeptin, 2 μM benzamidine), mixed with prewarmed stimuli for 2 minutes at 28°C, then mixed with AcA-GTP carrying ^{32}P at the α or γ position (1–2 μCi) and incubated for 3 minutes. Empirical tests of conditions ranging from 0.5 to 10 minutes and 0° to 30°C were used to arrive at 3 minutes and 28°C as conditions that gave reliable photoaffinity labelling. Membranes were recovered by centrifugation at 16,000 × g in a 1.5-ml tube for 10 minutes at 4°C. Pellets were resuspended in 20 μl of cross-linking buffer (in mM: 10 NaCl, 30 HEPES, pH 7.4, 5 $MgCl_2$, 0.1 EGTA, 1 benzamidine, 2 dithiothreitol, 1 mercaptoethanol), transferred onto aluminum foil on ice, and treated with a hand-held, unshielded ultraviolet lamp (254 nm) at 5 cm distance for 5 minutes. The membrane proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose and allowed to expose X-ray film overnight. This same blot was then used for immunostaining as described previously (McClintock et al., 1997; Xu et al., 1997). Enhanced chemiluminescence (Supersignal; Pierce, Rockford, IL) allowed the detection of antigen on film within minutes, rapidly enough that radioactive decay from ^{32}P-AcA-GTP did not interfere.

Immunoprecipitation

Aliquots of brain homogenates were thawed on ice and brought to 1× stimulation buffer (in mM: 200 NaCl, 50 MOPS, 2.5 $MgCl_2$, 1 DTT, 10 EGTA, 0.04 ATP, 0.01 GTP, 6.4 Ca^{2+}, 0.1% Lubrol, pH = 7.4). The homogenates were stimulated for 1 minute at room temperature with vehicle

(stimulation buffer), 3 μM GTP-γ-S, or a neurotransmitter mixture containing 1 μM of serotonin, dopamine, acetylcholine, GABA, and histamine, and 0.1 μM substance P. Antiserum P293 to lobPLCβ was added to a final concentration of 20 μg/ml and then immunoprecipitation and analysis by Western blotting was performed as described (Xu and McClintock, 1999).

Statistical tests

A t-test for dependent samples in STATISTICA for Windows, 1999 (Statsoft Inc. [1999], Tulsa, OK) was used to test for significant differences. Mean values are shown with their standard errors.

RESULTS

Distribution of α subunits in the brain

Immunoreactivity for $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ was remarkably similar. Immunoreactivity for each was found in virtually all of the compartments within the brain (Figs. 1–3). In each case, this immunoreactivity was absent when the primary antiserum was omitted and greatly reduced by preabsorption with antigen (Figs. 1D, 2D, 3C). We observed no variation in the immunoreactive structures across the nine brains used. The strongest immunoreactivity for each α subunit was in neuropil, which contain the majority of synapses in the crustacean brain. However, moderate immunoreactivity was apparent in neuronal cell bodies and the olfactory globular tract. Weak immunoreactivity was observed in other fiber tracts. Figures 1–3 show that the patterns of immunoreactivity observed were very similar for all three antisera. Illustrative examples showing results with only one antiserum, therefore, are shown in some later figures. The few differences observed between the three antisera are noted below.

Immunoreactivity was unambiguously detected in the following neuropil regions: accessory lobe (AN), olfactory lobe (ON), anterior medial protocerebral neuropil (AMPN), posterior medial protocerebral neuropil (PMPN), lateral antenna I neuropil (LAN), medial antenna I neuropil (MAN), antenna II neuropil (AntN), tegumentary neuropil (TN), and deutocerebral commissure neuropil (DCN). In addition, the central body (CB) was immunoreactive for $G_{\alpha i}$ and $G_{\alpha q}$. We have not been able to clearly identify the CB in sections reacted with the $G_{\alpha s}$ antiserum, so we draw no conclusion about the presence of the $G_{\alpha s}$ in the CB. Immunoreactivity in dense neuropils, such as the glomeruli of the ON and AN, gave the appearance of dense collections of spots of varying intensities (Figs. 4, 5). In less dense neuropils, such as the AMPN, PMPN, DCN, AntN, and the LAN, the immunoreactivity was characterized by a mixture of spots and threads. In the ON and the AN, the most intensely immunoreactive structures were the glomeruli, but fiber bundles associated with these glomeruli were also immunoreactive (Figs. 1C, 2B,C, 3B). The gaps between the AN and ON glomeruli contained little immunoreactivity, suggesting that the glial elements that fill these spaces contain much less $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ than the glomerular neuropil (see Fig. 5). In addition to these common features, there were interesting differences in the intensity of immunoreactivity for each of the three α subunits in the glomeruli of the AN and ON (Figs. 4, 5). In the spherical glomeruli of the AN, $G_{\alpha i}$ and $G_{\alpha q}$ immunoreactivity was stronger in the periphery than in the core, whereas $G_{\alpha s}$ immunoreactivity was stronger in the core (Fig. 4). Consistent intensity differences within the columnar glomeruli of the AN and the ON were also visible, but less striking (Fig. 5). To quantity these differences, ratios of the average fluorescence intensity of glomerular regions were calculated for individual glomeruli. In the columnar glomeruli of the AN, $G_{\alpha s}$ immunoreactivity was more intense in the base than in the cap or subcap (2.0 ± 0.5-fold difference, n = 5), whereas immunoreactivity for $G_{\alpha i}$ and $G_{\alpha q}$ was more uniform (1.2 ± 0.3-fold difference, n = 5, and 1.1 ± 0.2-fold difference, n = 4, respectively). In glomeruli of the ON, immunoreactivity for $G_{\alpha i}$ was stronger in the base than in the cap or subcap (2.3 ± 0.3-fold difference, n = 4), whereas $G_{\alpha q}$ immunoreactivity and $G_{\alpha s}$ immunoreactivity were more uniform (1.2 ± 0.3-fold difference, n = 5, and 1.2 ± 0.3-fold difference, n = 4, respectively).

Immunoreactivity for all three antisera was observed in all the neuronal cell body clusters that we could clearly and consistently identify: 6, 8, 9, 10, 11, 13, 14, 16, and 17. In these clusters, immunoreactivity for $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ was observed in every cell body. It was more intense at the cell surface, with weaker, punctate staining in the cytoplasm (Fig. 6). Immunoreactivity at the cell surface was also punctate. This staining is more easily observed in the larger neurons of clusters 9, 16, and 11 (Fig. 6A,B,D), but can also be seen in the small neurons of cluster 10 (Fig. 6C). The most immunoreactive structures in clusters 9 and 10 were the fascicles that form as the neurites join to become the tracts that innervate the ON and AN (Figs. 1C, 2B, 3B, 6C). Because nonneuronal elements are rare in cell body clusters in crustacean brains (Linser et al., 1997; Schmidt, 1997a), these strongly immunoreactive structures are most likely to be neurites.

Fiber tracts in the brain were also immunoreactive for $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$. The olfactory globular tract (OGT) was the most immunoreactive fiber tract and had densely packed immunoreactive fibers throughout (Fig. 7A, inset). This staining was particularly strong in sections showing the OGT passing caudal to the deutocerebral commissure (DC) (Fig. 7B), in sections where the OGT wrapped under the DC (Fig. 7C), and in sections where it passed by or through the OGT neuropil (OGTN) and extended into the region between the AN and ON (Fig. 7C,D, see also Figs. 1A–C, 2A–C, 3B). Fibers from the AN and ON could be seen entering into the OGT, but the strong immunoreactivity in the glomeruli of the AN and ON made it impossible to trace the OGT fibers back into the neuropil. Much weaker immunoreactivity was observed in the DC, the optic tract, and the circumesophageal connectives. In the DC, immunoreactivity was strongest at the surface of small oval cell bodies, but also included a weaker, reticulated pattern of broad strands (Fig. 8). We were not able to determine conclusively whether these cell bodies were continuous with the broad strands.

The specificity of the antisera for $G_{\alpha q}$ and $G_{\alpha s}$ in lobster tissues was confirmed in previous studies (McClintock et al., 1997; Xu et al., 1997). To demonstrate the specificity of the antiserum for lobster $G_{\alpha i}$, Western blots were performed. Immunoreactivity was detected only in a band of ~41 kDa (Fig. 9), near the predicted molecular weight of 40.8 kDa.

Neurotransmitters stimulate $G_{\alpha i}$

GTP photoaffinity labelling was used to test the ability of neurotransmitters to stimulate heterotrimeric G-proteins. In preliminary studies with brain homogenates, we observed

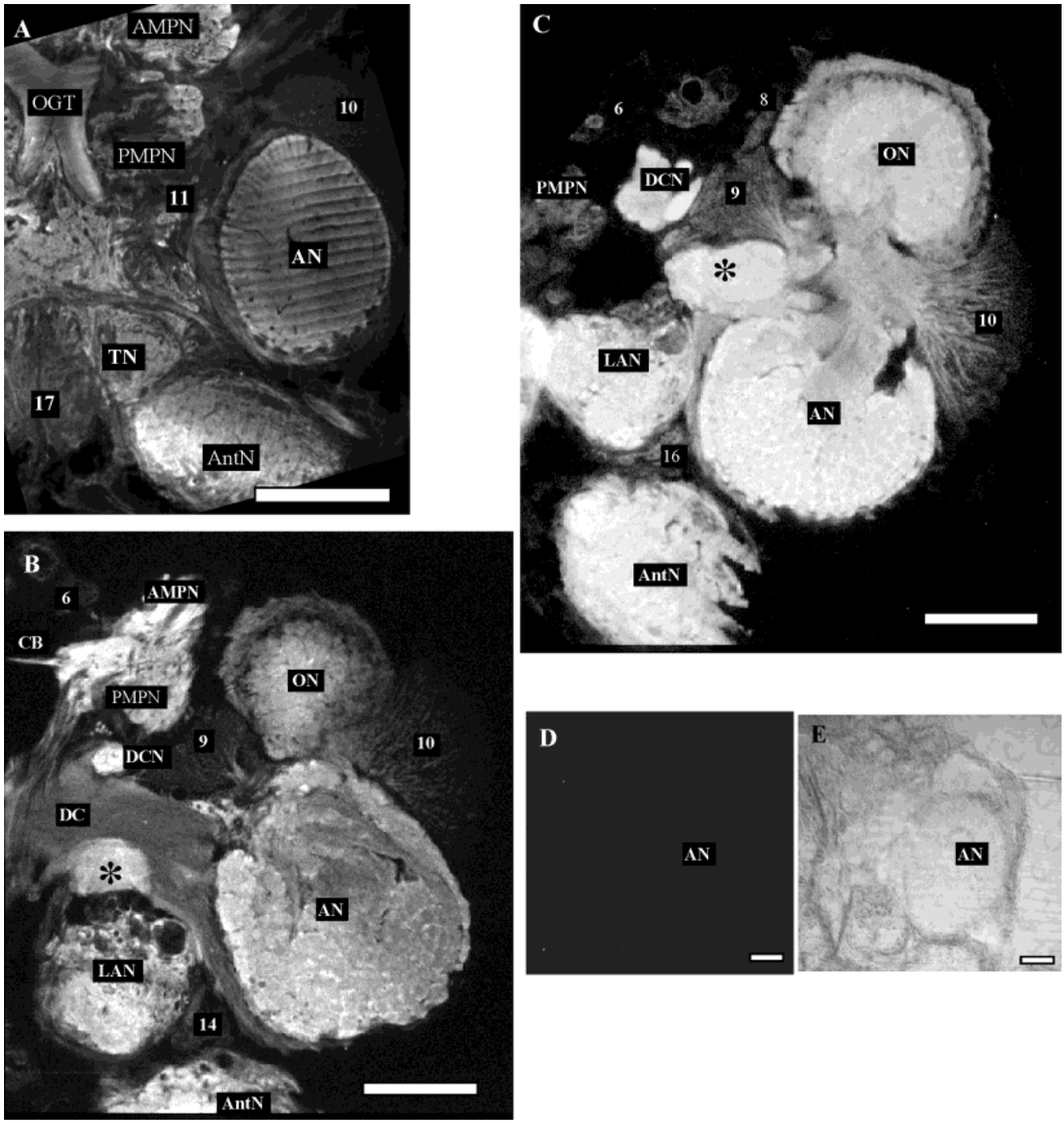


Fig. 1. Immunoreactivity for $G_{\alpha i}$. **A–C:** Three horizontal sections from the brain arranged dorsal to ventral. **D,E:** Fluorescent and brightfield images of the same portion of a horizontal section that was treated with antiserum C-10 preabsorbed with antigen to show that immunoreactivity was specific to the C-10 antigen. A lateral half of the bilaterally symmetric brain is shown in each panel; anterior is up, lateral to the right. Wide-field images are shown. Asterisks, OGT or OGT neuropil (OGTN); numbers, neuronal cell body clusters; AMPN, anterior medial protocerebral neuropil; AN, accessory lobe neuropil; AntN, antenna II neuropil; CB, central body; DCN, deutocerebral commissure neuropil, LAN, lateral antenna I neuropil; OGT, olfactory globular tract; ON, olfactory lobe neuropil, PMPN, posterior medial protocerebral neuropil; TN, tegumentary neuropil. The striations across the AN in panel A (and some panels of later figures) are Vibratome sectioning artifacts. Scale bars = 500 μm in A–C; 200 μm in D,E.

[^{32}P]AcA-GTP labelling of a single band that exactly matched in size the band immunoreactive for $G_{\alpha i}$ (Fig. 9). Even at longer preincubations with [^{32}P]AcA-GTP, labelling of bands at positions corresponding to immunoreactivity for $G_{\alpha q}$ and $G_{\alpha s}$ was weak. These observations are consistent with previous evidence that $G_{\alpha q}$ and $G_{\alpha s}$ are difficult to label

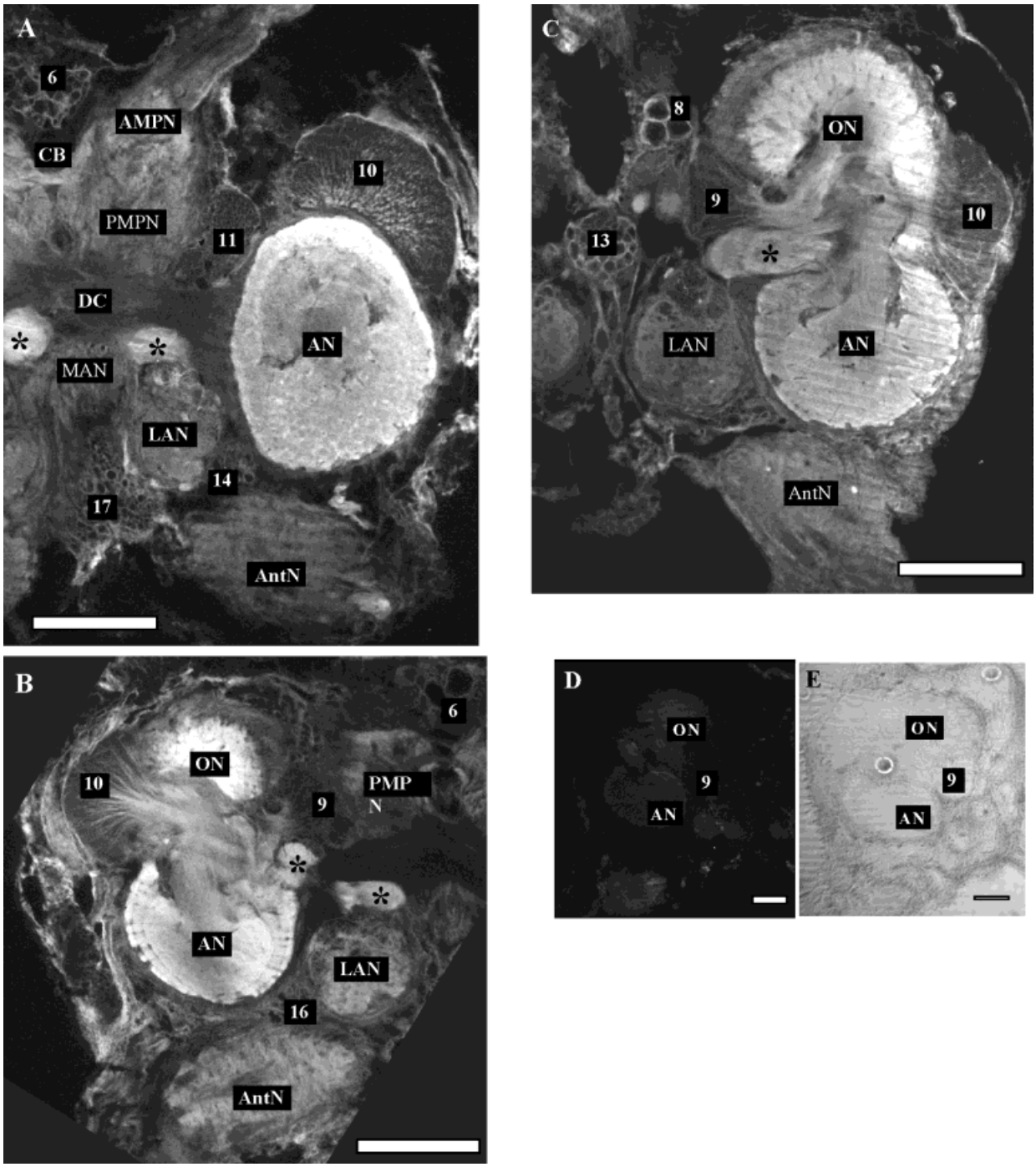


Fig. 2. Immunoreactivity for $G_{\alpha q}$. **A–C:** Three horizontal sections from the brain arranged dorsal to ventral. **D,E:** Fluorescent and brightfield images of the same portion of a horizontal section that was treated with antiserum C-19 preabsorbed with antigen to show that immunoreactivity was specific to the C-19 antigen. A lateral half of the bilaterally symmetric brain is shown in each panel; anterior is up, lateral to the right in A and C, to the left in B, D, E. Wide-field images are shown. Asterisks, OGT or OGTN. MAN, medial antenna I neuropil; For other abbreviations, see legend for Figure 1. Scale bars = 500 μm in A–C; 200 μm in D,E.

with photoaffinity analogs (Fields et al., 1994). We, therefore, focused our efforts on testing whether histamine, GABA, serotonin, substance P, and glutamate would stimulate $G_{\alpha i}$ in brain homogenates (Fig. 10). We observed increased AcA-GTP labelling of $G_{\alpha i}$ in response to histamine (0.1 μM, $t = 8.51$, $df = 2$, $P = 0.01$; 1 μM, $t = 5.16$, $df = 2$, $P = 0.03$; 10 μM, $t = 9.49$, $df = 2$, $P = 0.01$) but not in response to glutamate (0.1 μM, $t = 1.94$, $df = 2$, $P = 0.19$; 1 μM, $t = 0.56$, $df = 2$, $P = 0.63$; 10 μM, $t = 1.88$, $df = 2$, $P = 0.20$). We conclude that histamine activated $G_{\alpha i}$ but that glutamate did not. The other three substances also consistently increased labelling of $G_{\alpha i}$. The magnitudes of these responses

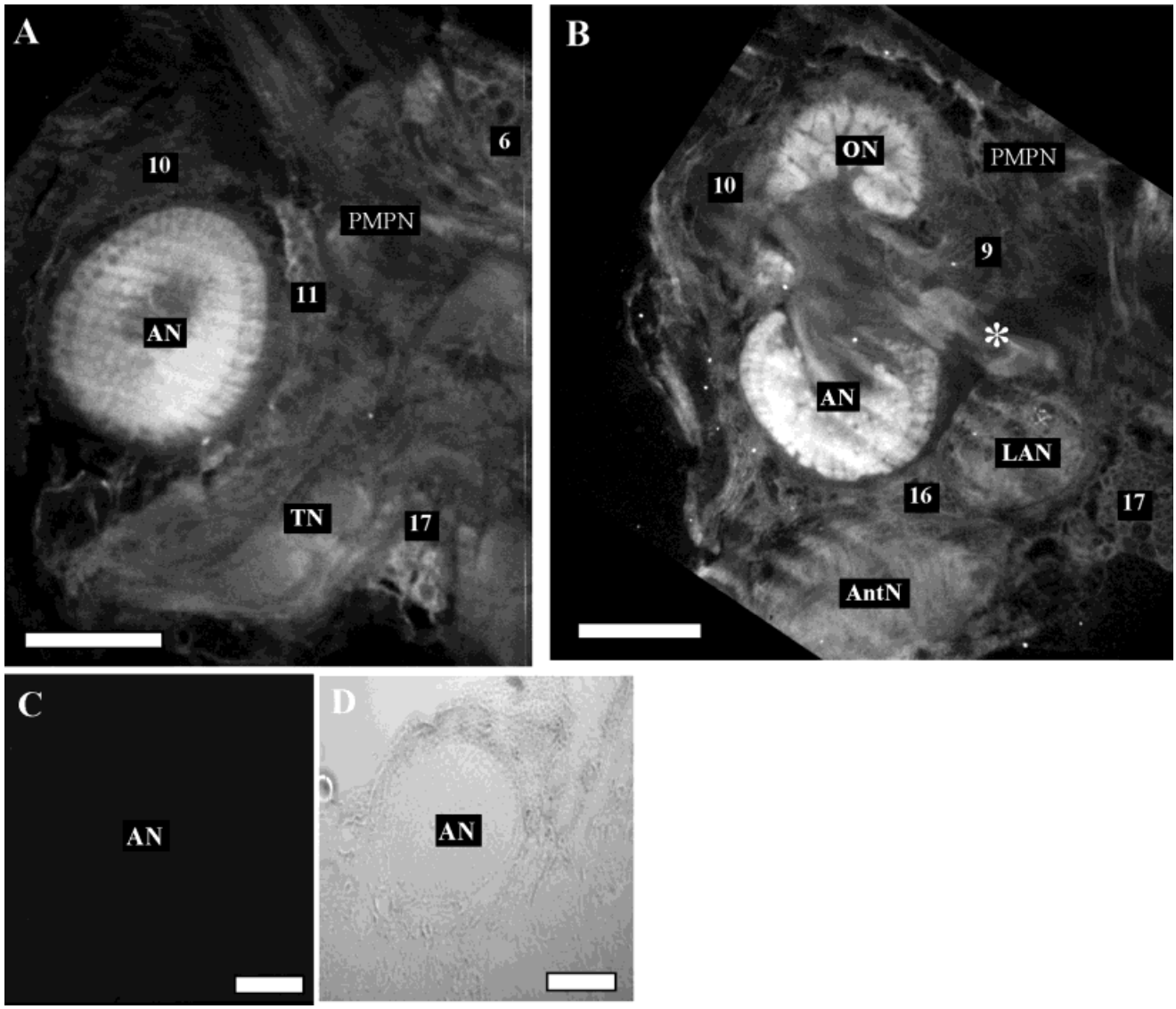


Fig. 3. Immunoreactivity for $G_{\alpha s}$. **A,B:** Two horizontal sections from the brain arranged dorsal to ventral. **C,D:** Fluorescent and brightfield images of the same portion of a horizontal section that was treated with antiserum C-18 preabsorbed with antigen to show that immunoreactivity was specific to the C-18 antigen. A lateral half of the bilaterally symmetric brain is shown in each panel, anterior is up, lateral to the left. Wide-field images are shown. Asterisk, OGT or OGTN. For abbreviations, see legend for Figure 1. Scale bars = 500 μm in A–C; 200 μm in D,E.

were more variable, however, and these data did not reach statistical significance: serotonin (10 μM, t = 2.49, df = 4, $P = 0.07$), GABA (10 μM, t = 2.10, df = 3, $P = 0.12$), and substance P (1 μM, t = 1.98, df = 2, $P = 0.19$).

Neurotransmitters stimulate association of lobPLCβ with $G_{\alpha q}$ and G_{β}

The generation of an antiserum to lobPLCβ (Xu and McClintock, 1999) provided a means of demonstrating that neurotransmitters have effects on $G_{\alpha q}$ in brain homogenates. Both $G_{\alpha q}$ and $G_{\beta\gamma}$ are known to stimulate β isoforms of phospholipase C by direct binding to conserved sites on the enzyme (Kim et al., 1996; Kuang et al., 1996; Yan and Gautam, 1997). Therefore, we predicted that lobPLCβ would increase its association with $G_{\alpha q}$ and G_{β} in response to neurotransmitters. Indeed, stimulation of lobster brain membrane preparations with a neurotransmitter mixture or with GTP-γ-S increased the amount of lobPLCβ immunoreactivity in immunoprecipitates of $G_{\alpha q}$ and G_{β} (Fig. 11A–C). In G_{β} immunoprecipitates, the increases were fourfold and sixfold in response to the neurotransmitter mixture and GTP-γ-S, respectively. In $G_{\alpha q}$ immunoprecipitates, the increases were 5-fold and 11-fold, respectively. Consistent with these observations, we found that the neurotransmitter mixture and GTP-γ-S also stimulated translocation of lobPLCβ from the soluble fraction to the membrane fraction of brain homogenates (Fig. 11D,E, 12).

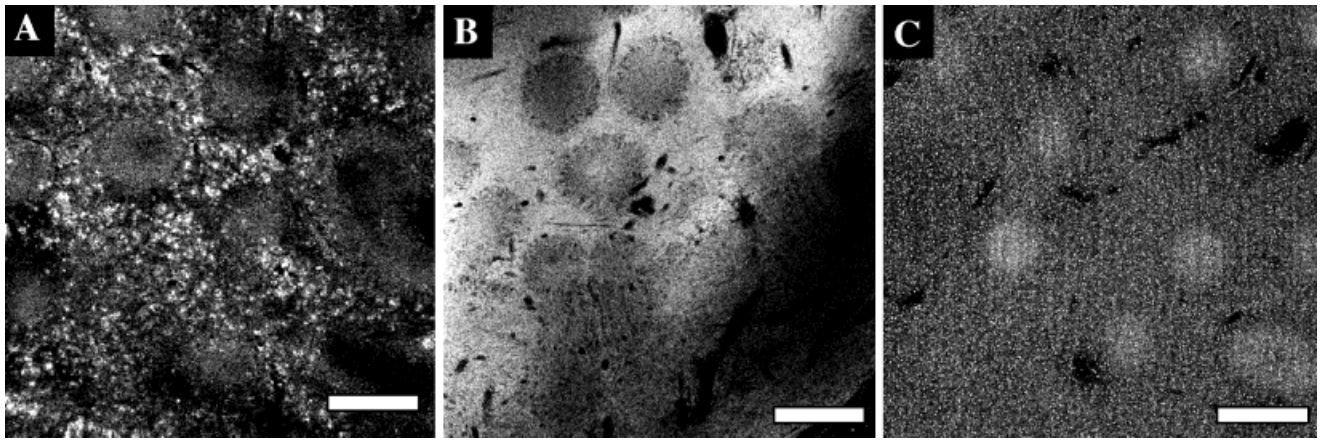


Fig. 4. In the spherical glomeruli of the AN, immunoreactivity for $G_{\alpha i}$ (**A**) and $G_{\alpha q}$ (**B**) was stronger in the periphery than in the core, whereas immunoreactivity for $G_{\alpha s}$ (**C**) was stronger in the core than in the periphery. Confocal images from horizontal sections are shown. Scale bars = 20 μm.

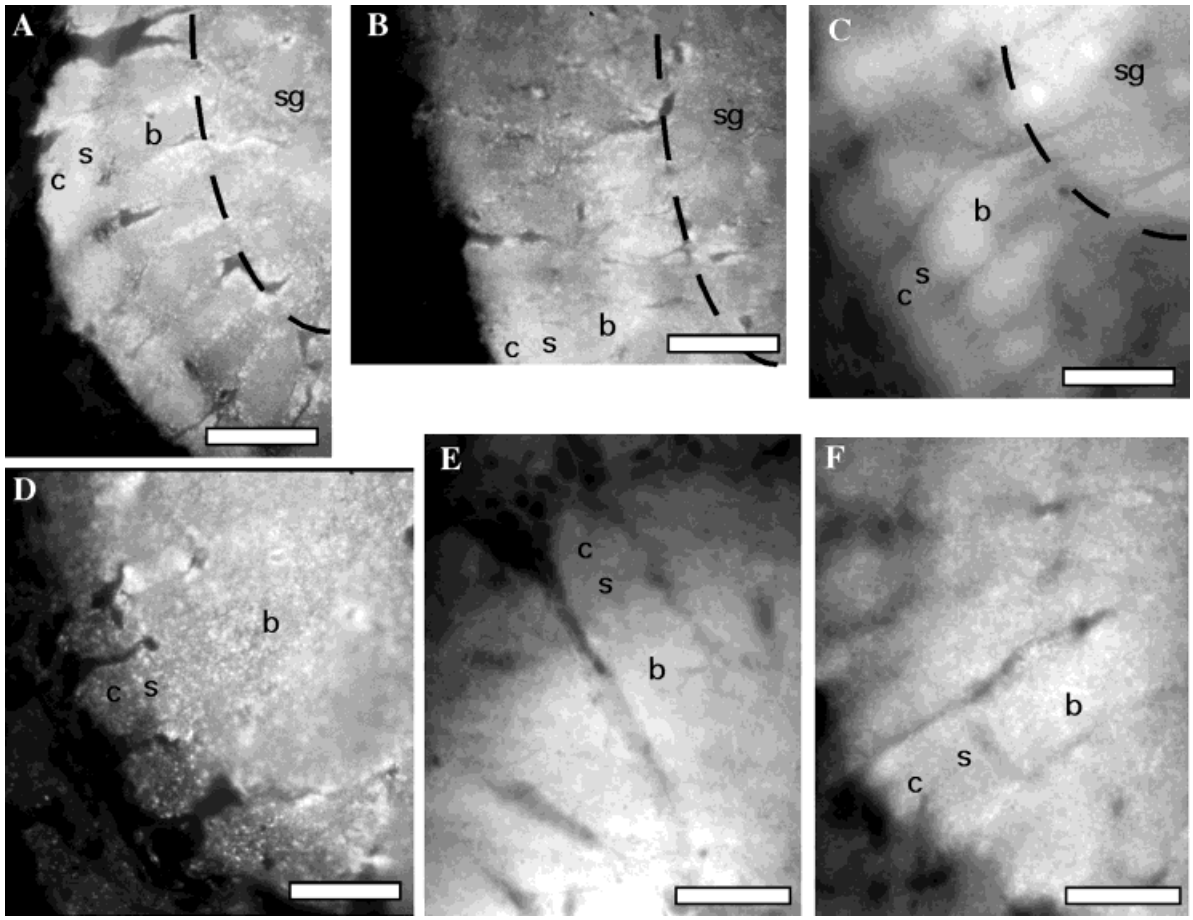
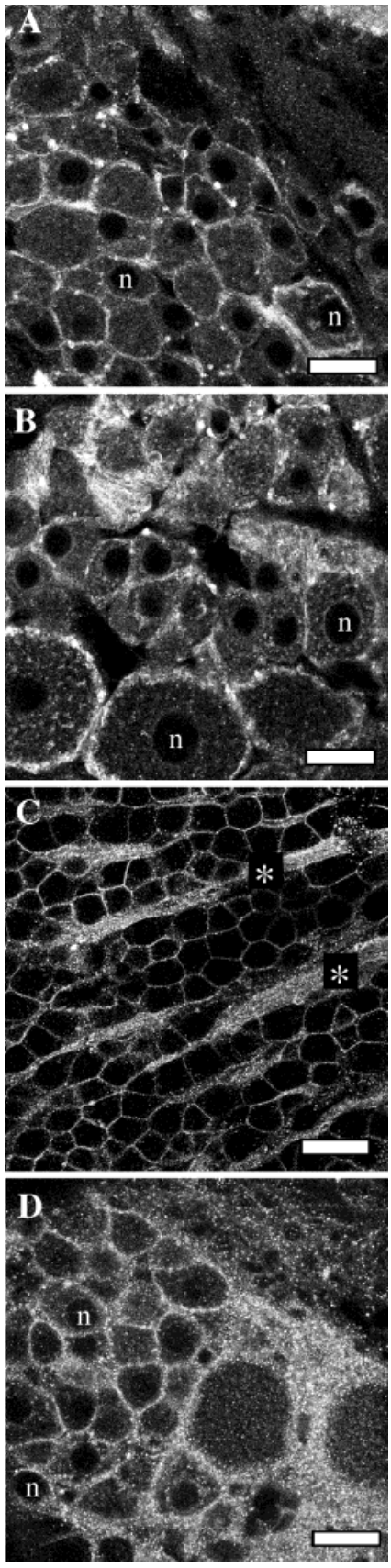


Fig. 5. Immunoreactivity in the columnar glomeruli of the AN (A–C) and the ON (D–F). **A,D:** $G_{\alpha i}$ immunoreactivity. **B,E:** $G_{\alpha q}$ immunoreactivity. **C,F:** $G_{\alpha s}$ immunoreactivity. The paucity of immunoreactivity in the gaps between glomeruli where glial cells reside is easily observed in these images. Wide-field images of horizontal sections are shown. b, base; c, cap; s, subcap; sg, spherical glomeruli. Scale bars = 50 μm.

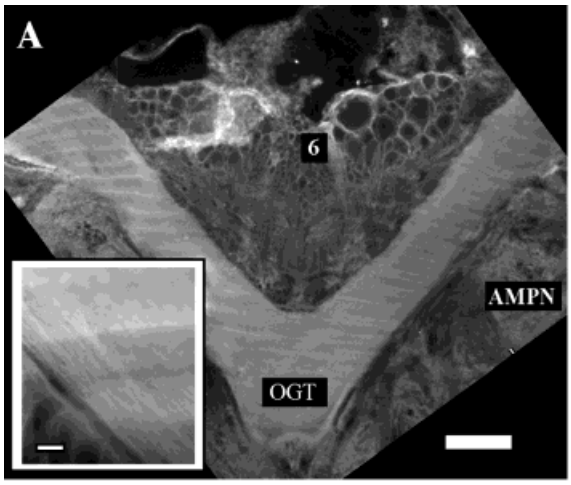


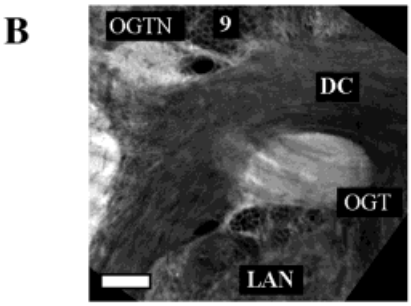
DISCUSSION

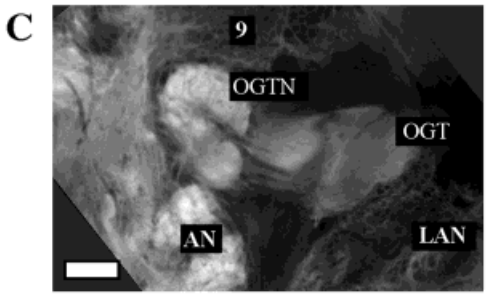
Immunoreactivity for $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ was widespread in the lobster brain and had common patterns of intensity: neuropil > cell body clusters ≥ fiber tracts. For the AN and ON, at least, neuropil staining was most intense in areas exclusively containing neurites and least intense in areas that contain glia (Linser et al., 1997). The intense neuropil staining and its punctate nature is consistent with the concentration of synapses in arthropod neuropil. Both pre- and postsynaptic elements could contribute to this pattern. The $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ immunoreactivity at the cell surface of neuronal cell bodies is consistent with evidence of axons containing neuroactive substances innervating crustacean cell body clusters (Sandeman et al., 1990; Langworthy et al., 1997; Schmidt, 1997b), and with evidence that neuronal cell bodies from cluster 10 are modulated by neurotransmitters (Schmidt, 1997b; Wachowiak and Ache, 1998). Expression in the brain may not be limited to neurons, however. Our results indicate only that expression in neurons, especially neurites, is much greater than in glia. As discussed below, the relatively weak immunoreactivity in the DC may represent expression in glia. We conclude that $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ are coexpressed in most, if not all, neurons of the brain and probably coexist in their dendrites as well as their cell bodies.

Our discovery that the OGT was moderately immunoreactive for $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ is intriguing because fiber tracts are thought to lack synaptic connections. The pattern of OGT immunoreactivity, which was in small fibers tightly packed together and attached to the AN and ON glomeruli, differs from the pattern of weak immunoreactivity in the DC, which included small oval cell bodies and a reticulated network of broad strands. We believe that the fine fibers in the OGT represent neurites and that the broad strands in the DC represent glia. The space in fiber tracts in the crustacean brain is dominated by fascicles of unmyelinated fibers, which are separated by thin extensions of glial cells with occasional glial cell bodies interspersed (cf. Fig. 2 in Schmidt, 1997a). The OGT immunoreactivity we observed matched the anatomy of the fibers, whereas the DC immunoreactivity matched the anatomy of the glial elements. That glial elements contributed a minor portion of the immunoreactivity in the OGT is also possible. Likewise, some immunoreactivity in the DC may be neuronal. Several possible explanations for the presence of significant amounts of $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ in the OGT exist. (1) OGT immunoreactivity could indicate that concentrations of synapses occur along a large part of the OGT, much larger than the previously circumscribed OGTN. (2) Axons in the OGT could have concentrations of nonsynaptic signalling pathways involving $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$. (3) The immunoreactivity could represent G-protein subunits being transported down axons to synaptic sites in distant neuropils. That synapses have not previously

Fig. 6. Immunoreactivity in representative neuronal cell body clusters. **A:** $G_{\alpha i}$ immunoreactivity in cluster 9. **B:** $G_{\alpha i}$ immunoreactivity in cluster 16. **C:** $G_{\alpha q}$ immunoreactivity in cluster 10. **D:** $G_{\alpha s}$ immunoreactivity in cluster 11. Confocal images from horizontal sections are shown. Asterisks, immunoreactivity in the fascicles that join into the tract that innervates the ON and AN; n, nucleus. Scale bars = 30 μm.







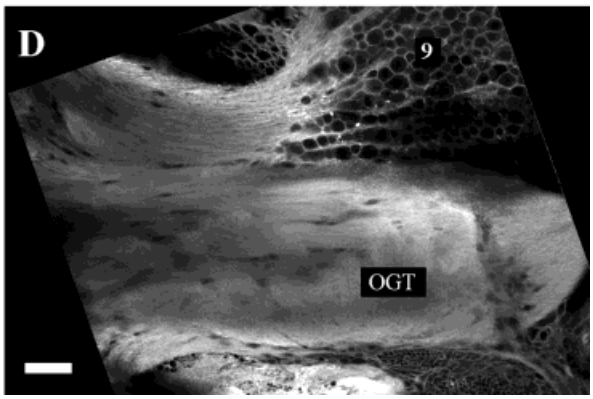


Fig. 7. $G_{\alpha q}$ immunoreactivity in the OGT and OGTN in horizontal sections arranged from dorsal to ventral. **A:** Dorsal sections (wide-field) show uniform immunoreactivity in the OGT as it approaches the protocerebral tract at the anterior end of the brain. **Inset:** high magnification, wide-field view showing bundles of fine fibers in the OGT. **B:** Near the midline of the brain, a horizontal section (wide-field) shows the OGT as it passes caudal to the DC, which separates it from part of the OGTN at this level. **C:** More ventrally, the OGT passes under the DC and connects with the OGTN. A wide-field image is shown. **D:** Further ventrally, tight bundles of OGT fibers project between the AN and ON. A confocal image is shown. The striations across the OGT in A and B are Vibratome sectioning artifacts. Anterior is up in all panels, lateral to the left in B–D. Scale bars = 20 μm in A; 100 μm in B,C; 25 μm in D.

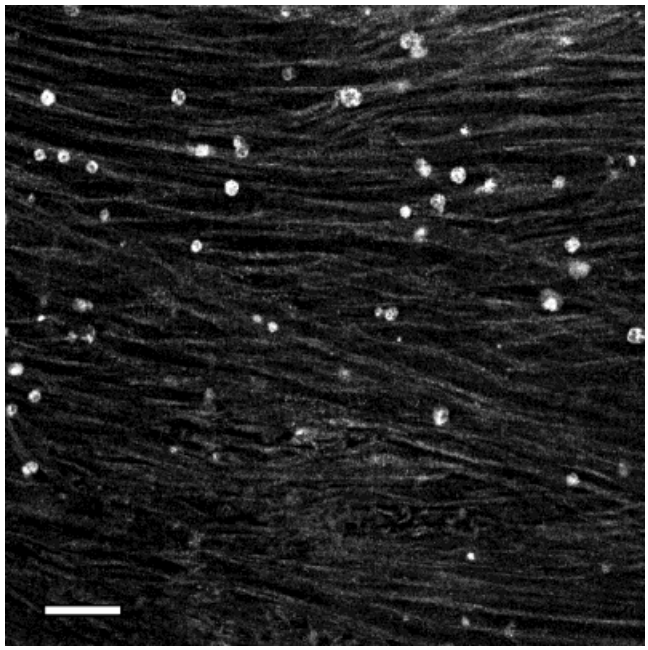


Fig. 8. A confocal image of $G_{\alpha i}$ immunoreactivity in the DC showing the weakly immunoreactive broad strands that form a reticulated pattern and strongly immunoreactive small cell bodies. Scale bar = 20 μm.

been observed in the OGT favors that the second and third explanations.

The differences in intensity of immunoreactivity for a given G-protein α subunit in subregions of ON and AN glomeruli are of interest for several reasons. First, the ON and AN are the dominant features of the crustacean deutocerebrum. In *H. americanus*, the ON and AN have a similar organization, with a cortex of columnar glomeruli that have an thin external cap region over a slightly thicker subcap and an elongated base. Deep to these columns are spherical glomeruli. The ON is dedicated to processing olfactory signals and receives the afferent fibers from the olfactory receptor neurons that reside in the lateral filament of the first antenna (Schmidt and Ache, 1992). The AN is a center for processing multimodal information, including vision, mechanosensation, and olfaction (Sandeman et al., 1995). Second, many neuronal types that innervate the AN and ON are known to branch

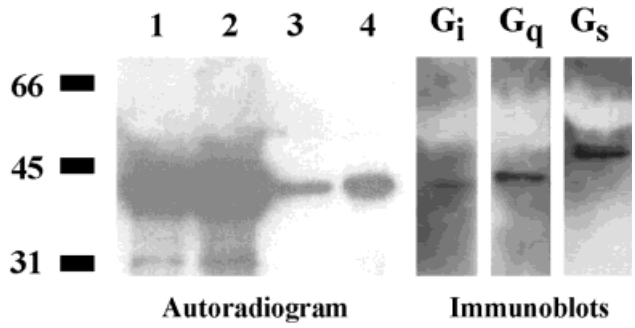


Fig. 9. Determination that $G_{\alpha i}$, but not $G_{\alpha q}$ or $G_{\alpha s}$, was labeled by a photoaffinity analog AcA-GTP. An autoradiogram from nitrocellulose blot of an sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel shows that a single band of protein was strongly labeled by AcA-GTP in brain homogenates under different conditions. **Lane 1:** preincubation for 10 minutes at 30°C. **Lane 2:** preincubation for 30 minutes at 30°C. **Lane 3:** preincubation for 10 minutes at 1°C. **Lane 4:** preincubation for 30 minutes at 1°C. The lanes were then separated by cutting the nitrocellulose filter and used for Western blots. Lane 2 used for $G_{\alpha q}$ Western blot; lane 3, $G_{\alpha i}$; lane 4, $G_{\alpha s}$.

selectively in certain subregions of glomeruli (Schmidt and Ache, 1992; Langworthy et al., 1997). Third, these anatomically defined subregions are neurochemically distinct (Orona et al., 1990; Schmidt and Ache, 1992, 1994a, 1997; Wachowiak and Ache, 1994; Sandeman et al., 1995; Wachowiak et al., 1996; Langworthy et al., 1997). For example, in *H. americanus* the cap of ON glomeruli contains immunoreactivity for GABA, histamine, and serotonin, the subcap serotonin, small cardioactive peptide b, and substance P, and the base serotonin, histamine, and small cardioactive peptide b (Langworthy et al., 1997). Our results contribute evidence that synaptic signal transduction pathways also show differential distributions. $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ each showed evidence of differential distribution among the subregions of either AN or ON glomeruli, or both. Several lines of evidence, therefore, converge on the prediction that the glomerular subregions of the AN and ON are segregated sites of synaptic functions that regulate the processing of sensory information in the crustacean brain.

For functional experiments, we focused primarily on neuroactive substances that are found in, or have neuromodulatory effects on, the ON and AN: serotonin, dopamine, substance P, histamine, GABA, acetylcholine, and glutamate (Orona et al., 1990; Langworthy et al., 1997; Schmidt and Ache, 1997; Wachowiak and Ache, 1998). Our results confirm that $G_{\alpha i}$ and $G_{\alpha q}$ are responsive to at least some of these neuroactive substances. A mixture of a subset of these neurotransmitters stimulated the association of lobPLCβ with $G_{\alpha q}$, G_{β}, and membranes. We previously observed virtually identical results when stimulating olfactory dendrite homogenates with odorants and eye homogenates with light (Xu and McClintock, 1999). These interactions are known to be stimulatory for β isoforms of PLC by directly influencing catalytic activity and by bringing PLCβ in contact with its substrate in the plasma membrane (Jiang et al., 1994; Lee et al., 1994; Lee and Rhee, 1995). In experiments with single stimuli, we demonstrated that histamine is capable of stimulating $G_{\alpha i}$ in homogenates of brain. Similar, or even greater, stimulation of $G_{\alpha i}$ was caused by substance P, serotonin, and GABA, but the greater variability of these responses precluded firm conclusions about these effects. The three substances can stimulate $G_{\alpha i}$ in mammalian cells (Watson and Arkinstall, 1994), and recent evidence indicates that

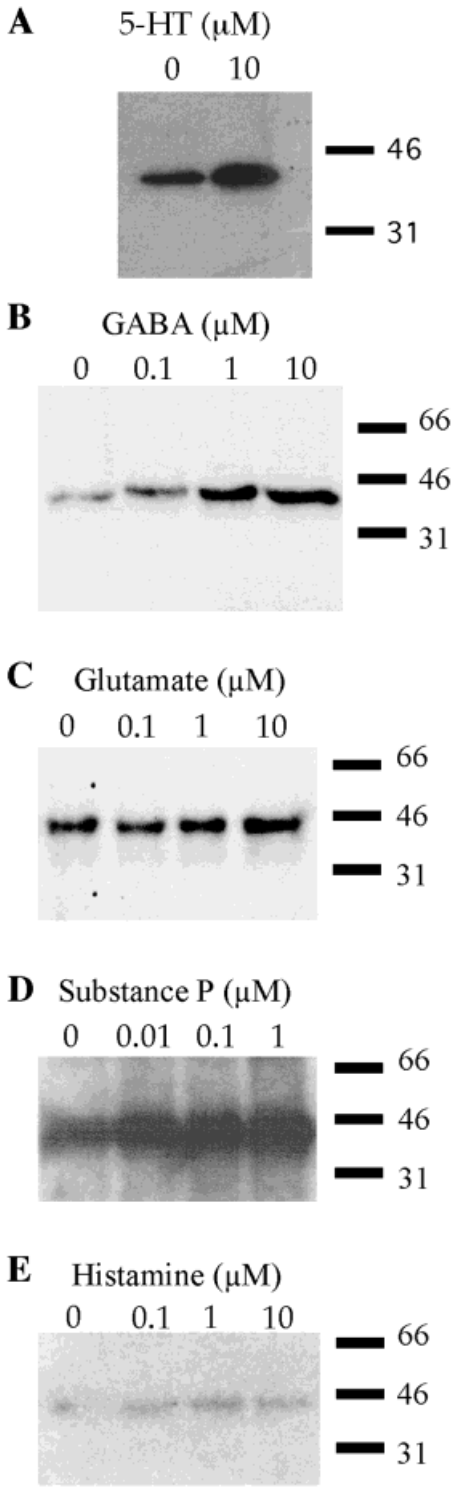
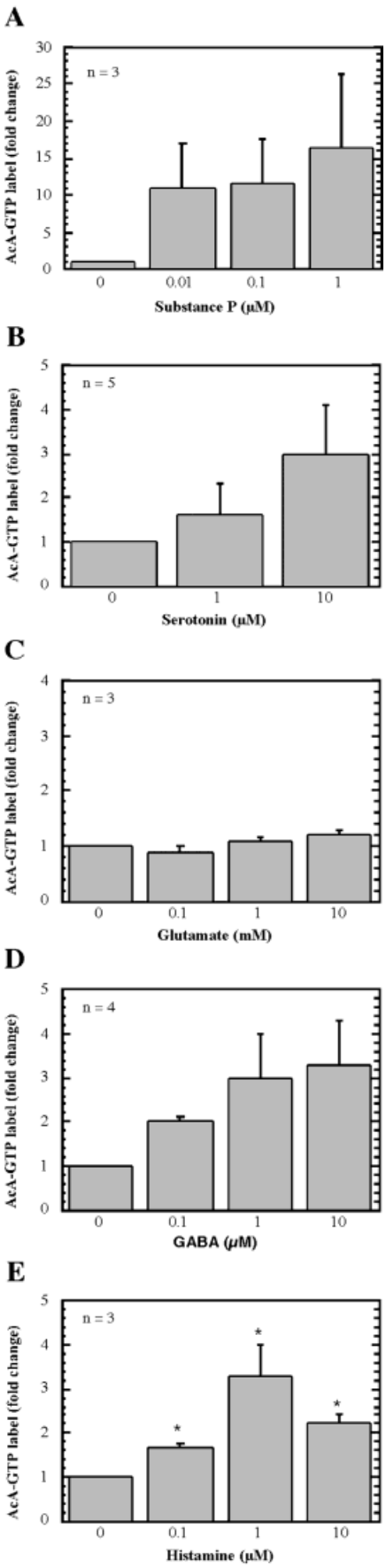


Fig. 10. Stimulation of $G_{\alpha i}$ in lobster brain homogenates by serotonin (5-HT) (**A**), GABA (**B**), substance P (**D**), and histamine (**E**) was detected by autoradiography of [^{32}P]AcA-GTP photoaffinity labelling. Glutamate (**C**) had little effect.



histamine can also (Takeshita et al., 1998). Our conclusion that histamine is merely capable of activating $G_{\alpha i}$ is necessarily cautious because some G-protein coupled receptors can activate G-proteins promiscuously in homogenates (e.g., Laugwitz et al., 1996; Allgeier et al., 1997), and because the disruption of subcellular compartments could allow interactions of receptors and G-proteins that do not exist in vivo. The lack of response to glutamate suggests that if metabotropic glutamate receptors occur in the lobster brain, they are not capable of coupling to $G_{\alpha i}$. This differs from mammals, which have several isoforms of metabotropic glutamate receptors that primarily activate $G_{\alpha i}$ and are expressed in brain (Pin and Duvoisin, 1995). Perhaps a response to glutamate would have been observed at higher concentrations, but 10 μM is sufficient to cause maximal inhibition of adenylyl cyclase (Duvoisin et al., 1995) and near-maximal stimulation of PLC (Abe et al., 1992) by mammalian metabotropic glutamate receptors. An alternative explanation is that lobster glutamate receptors were desensitized by the release of glutamate from the tissue homogenate itself. We cannot completely rule out this possibility, though we purposefully diluted the homogenates to reduce this problem and were successful in retaining capacity for responses from receptors to the other neuroactive substances.

Previous studies have shown that $G_{\alpha q}$ and $G_{\alpha s}$ are more difficult to label than $G_{\alpha i}$ because they more rapidly hydrolyze photoaffinity analogs (Fields et al., 1994). Our results were consistent with this finding, and prevented us from using this method to investigate the capacity of neuroactive substances to stimulate $G_{\alpha q}$ and $G_{\alpha s}$ in lobster brain homogenates. However, we did observe that when brain homogenates were stimulated with neurotransmitters such as octopamine and serotonin, the amount of $G_{\alpha q}$ and $G_{\alpha s}$ in the plasma membrane fraction of the homogenate was rapidly reduced. This behavior has been observed previously in mammalian cell homogenates, and is due to activation-dependent depalmitoylation of $G_{\alpha q}$ and $G_{\alpha s}$ (Klinz and Costa, 1990; Levis and Bourne, 1992; Mumby et al., 1994; Wedegaertner and Bourne, 1994; Stanislaus et al., 1997).

Our evidence indicates that the lobster brain is richly endowed with neuromodulatory biochemical pathways involving $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$. The robust immunoreactivity in neuropil and our initial functional studies suggest that a major role for these G-proteins is to mediate signal transduction for a wide variety of neuroactive substances that participate in neural processing in the lobster brain. This interpretation is consistent with evidence from physiologic studies showing that complex modulatory responses to neuroactive substances regulate the function of networks of crustacean CNS neurons (rev. by Marder et al., 1998; Selverston et al., 1998; Beltz, 1999).

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Fig. 11. Average changes in photoaffinity labelling of $G_{\alpha i}$ in lobster brain homogenates in response to substance P (**A**), serotonin (**B**), glutamate (**C**), GABA (**D**), and histamine (**E**). Error bars, standard errors of the mean.

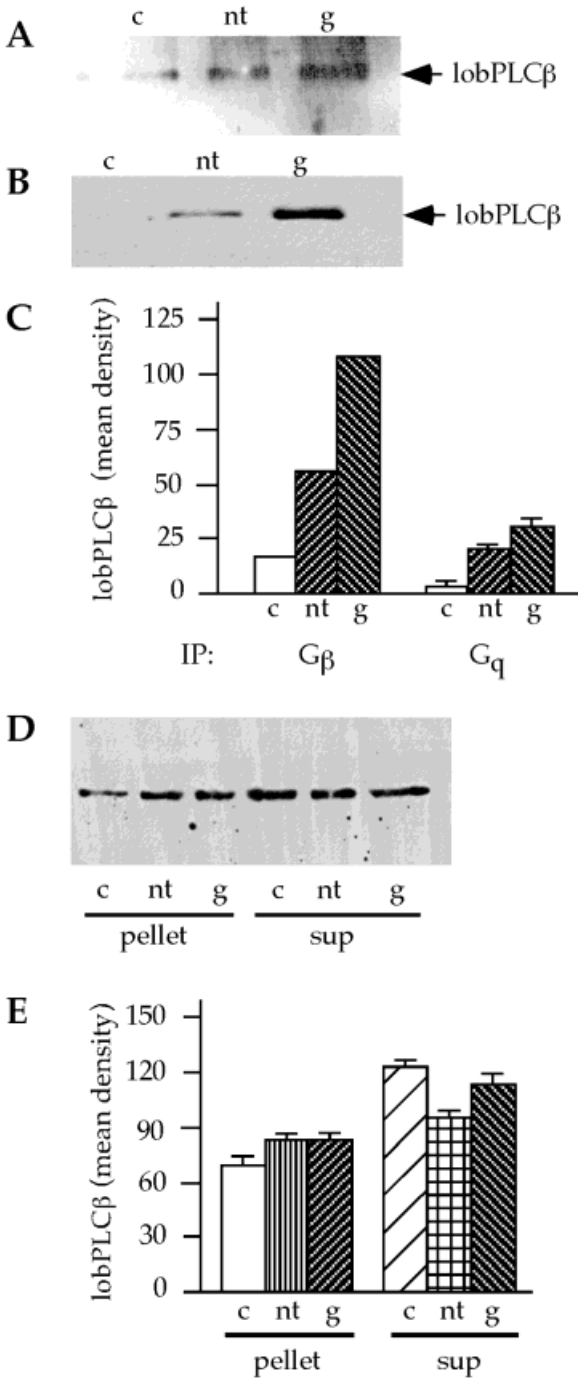

Fig. 12. GTP-γ-S or a mixture of neurotransmitters increased the association of lobPLCβ with $G_{\alpha q}$, G_{β}, and membrane fractions in tissue homogenates from the brain. **A:** Immunoprecipitation with G_{β} antisera precipitated lobPLCβ immunoreactivity. **B:** Immunoprecipitation with $G_{\alpha q}$ antisera precipitated lobPLCβ immunoreactivity. **C:** Densitometry of lobPLCβ immunoreactivity in the immunoprecipitates. $G_{\alpha q}$ immunoprecipitates, n =2; G_{β} immunoprecipitates, n = 1. **D:** LobPLCβ immunoreactivity shifted from the supernatant to the membrane pellet after stimulation. Detection of lobPLCβ by Western blot. **E:** Densitometry of data shown in D (n = 2). c, vehicle control; nt, neurotransmitter mixture (1 μM each of serotonin, dopamine, acetylcholine, GABA, and histamine, and 0.1 μM substance P); g, 3 μM GTP-γ-S; sup, supernatant fraction; IP, immunoprecipitates.

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