0022-3565/01/2993-840–848
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 299:840–848, 2001

Vol. 299, No. 3
4185/944660
Printed in U.S.A.

Colocalization of μ-Opioid Receptors and Activated G-Proteins in Rat Cingulate Cortex

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Received May 30, 2001; accepted August 15, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Anterior cingulate cortex (ACC) has a role in pain processing, however, little is known about opioid system organization and actions. This rodent study defines opioid architecture in the perigenual and midcingulate divisions of ACC, relates μ-opioid receptor binding and G-protein activation, and localizes such binding to afferent axons with knife-cut lesions and specifically to noradrenergic terminals with immunotoxin lesions (anti-dopamine β-hydroxylase-saporin; anti-DBH-saporin). [^{3}H]Tyr-D-AlaGly-MePhe-Gly-ol (DAMGO) binding was highest in perigenual areas 32 and 24 with a peak in layer I. Midcingulate area 24′ and posterior cingulate area 29 had overall lower binding in each layer. In contrast, DAMGO-stimulated [^{35}S]guanosine-5′-*O*-(γ-thio)-triphosphate (GTPγS) binding in area 24′ was similar to that in area 24, whereas area 29 had low and homogeneous binding. Undercut lesions reduced [^{3}H]DAMGO binding in all layers with the greatest loss in layer I (−65%), whereas DAMGO-stimulated [^{35}S]GTPγS binding losses occurred in only layers I-III. Anti-DBH-saporin reduced [^{3}H]DAMGO binding in layer I of area 24; DAMGO-stimulated [^{35}S]GTPγS binding was unchanged in areas 24′ and 29. Correlation analysis of receptor and G-protein activation before and after undercut lesions suggested there were a greater number of DAMGO receptor sites for each G-protein on axons, than on somata and proximal dendrites. Finally, perigenual and midcingulate cortices have different opioid architectures due to a higher proportion of μ-opioid receptors expressed by afferent axons in areas 24 and 32.

A primary cortical site of opiate drug actions is anterior cingulate cortex (ACC). Although much of this binding is associated with μ-opioid receptors (MOR) shown with high-capacity binding of carfentanil, there is also a high level of δ-opioid receptor binding (Frost et al., 1990). Coregistration of diprenorphine binding capacity with magnetic resonance images shows that perigenual cingulate areas 25, 32, and 24 have the highest binding in the cingulate gyrus, midcingulate areas 24a′/b′ and 32′ have a high capacity but the sulcal areas 24c′/24d have low binding, and binding is generally low in posterior cingulate cortex (Vogt et al., 1995a). Because acute noxious stimuli activate the midcingulate areas (Casey et al., 1994; Coghill et al., 1994; Vogt et al., 1996) and this may be associated with the motivational aspects of pain, one of the primary sites by which pain processing is regulated by opiate compounds is through binding to opioid receptors in midcingulate cortex. In addition, pain processing and opiate binding also occurs in the perigenual part of ACC. To the extent that this latter region is involved in the affective component of pain (Vogt et al., 1996) and morphine elevates blood flow in perigenual cortex (Jones et al., 1991), both motivational and affective components of pain mediated by ACC are modulated by opiate drugs.

The involvement of ACC in chronic pain has been assessed in terms of activity in the opioid system. Reductions of binding capacity for diprenorphine may be a consequence of elevated activity in opioidergic neurons and hence enhanced occupancy of the opioid receptors. Using this technique, it has been shown that ACC has significant elevations in opioid function in atypical facial pain (Derbyshire et al., 1994), rheumatoid arthritis (Jones et al., 1994), and trigeminal neuralgia (Jones et al., 1999).

The mechanisms by which MOR regulate pain in ACC are poorly understood. These receptors are expressed by cortical neurons largely in layer V and thalamic afferent axons, particularly in layer I, although both populations are found in all

This work was supported by Cingulum NeuroSciences Institute, Department of Veterans Affairs Medical Research Service (to R.G.W.) and Public Health Service Grants NS38485 (to B.A.V.), DA00287 (to L.J.S.-S.), and DA02904 (to S.R.C.).

ABBREVIATIONS: Anti-DBH-Saporin, anti-dopamine β-hydroxylase conjugated saporin; DAMGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; ACC, anterior cingulate cortex; GTPγS, guanosine-5′-*O*-(γ-thio)-triphosphate; MOR, μ-opioid receptor; ANOVA, analysis of variance.

layers (Vogt et al., 1995b). Although the endomorphins may be endogenous ligands for MOR (Monory et al., 2000), there are only rare endomorphin-immunoreactive processes in ACC (Martin-Schild et al.,1999). Therefore, met-enkephalinergic neurons appear to be the primary source of peptide that activates MOR and met-enkephalinergic neurons are found throughout ACC (Sar et al., 1978; Khachaturian et al., 1983).

The development of an agonist-stimulated [^{35}S]GTPγS binding assay for opioid receptors (Traynor and Nahorski, 1995) and an autoradiographic technique to visualize such binding (Sim et al., 1996) provides a strategy for analyzing coupling mechanisms by which MOR function is transduced in ACC. Studies of μ-opioid ligand binding and stimulated G-proteins in the same brains provide a means of determining the extent to which the density of each is coupled. Thus, Maher et al. (2000) assessed Scatchard analysis of antagonist binding and a full range of GTPγS concentrations and reported the ratio of opioid receptors/GTPγS-stimulated binding ranged between 1:8 in the thalamus to 1:40 in sensorimotor cortex, whereas there was an intermediate ratio in frontal cortex of 1:20.

Noradrenergic terminals in cingulate cortex may express MOR and provide for interactions between the opioid and adrenergic systems. Neurons in the locus coeruleus synthesize μ-opioid and α-adrenoceptors (Delfs et al., 1994; Mansour et al., 1994), both interact with G_i/G_o proteins (Limbird, 1988), hyperpolarize neurons in the locus coeruleus, reduce norepinephrine release from axon terminals and ligands for both receptors increase K+ efflux (Zimanyi et al., 1988), and reduce Ca^{2+} influx (Seward et al., 1991). Noradrenergic projections to cingulate cortex are more pronounced than to lateral neocortical areas, and they are most dense in layer I (Morrison et al., 1978) where most axonal MOR are also located.

Finally, it has been proposed that, like primate ACC, the rodent ACC has perigenual and midcingulate parts (Vogt, 1993) based on circuit and functional considerations. Thus, the mediodorsal thalamic nucleus and amygdala project mainly to areas 24 and 32 and less in area 24′, whereas area 24′ has a strong pontine projection not characteristic of area 24. Opioid systems have not been considered in the context of structural and functional heterogeneity of ACC. The present study has three goals: 1) Describe relations of MOR binding and μ-receptor-activated G-proteins in perigenual, midcingulate, and posterior cingulate corticies; 2) evaluate these relations after undercut lesions in ACC; and 3) test the hypothesis that MOR are expressed by noradrenergic terminals.

Experimental Procedures

Materials. Male, Long Evans rats (350–400 g) were purchased from Harlan Labs (Indianapolis, IN). [^{35}S]GTPγS (specific activity, 1250 Ci/mmol), [^{3}H]DAMGO (specific activity, 55.3 Ci/mmol), and Reflections autoradiography film were purchased from PerkinElmer Life Sciences (Boston, MA). Hyperfilm β-max was obtained from Amersham Life Sciences (Arlington Heights, IL). NTB2 autoradiography emulsion was purchased from Kodak (Rochester, NY). DAMGO, naloxone, and GDP were purchased from Sigma (St. Louis, MO).

Lesions. Rats were anesthetized with Chloropent (0.2 ml/l00 g body weight i.p.; concentration is 42.5 mg/ml chloral hydrate, 8.86 mg/ml pentobarbital) and then received either unilateral undercut lesions as previously described (Vogt et al., 1995b) or anti-DBH-saporin injections (Wrenn et al., 1996). All animal procedures were in strict accordance with approved animal care protocols employing The National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, undercut lesions were made by passing a scalpel blade 1.6 mm lateral to the midline, 4.5 mm ventral to the cortical surface of the brain extending from 1.7 mm rostral to bregma to 1.0 mm posterior to bregma. Coronal knife cuts were made at the rostral and caudal limits of the knife cuts to assure complete removal of afferent axons (n = 6). Because callosal axons do not express μ-opioid receptors, the contralateral hemispheres were used as controls. Six rats received 7 μg of anti-DBH-saporin injected into the left lateral ventricle. Control rats received vehicle injections. After a 2-week survival for undercut or 32 days for anti-DBH-saporin, animals were sacrificed by decapitation, and the brains were removed, frozen in isopentane, and stored at −80°C. Coronal, 20 μm-thick sections were cut throughout the rostrocaudal extent of the cingulate cortex at −20°C in a cryostat and thaw-mounted onto chrome-alum subbed slides. Slides were desiccated at 4°C overnight and then stored at −80°C.

Autoradiography. Two autoradiographic techniques were employed in these studies. Film autoradiography was used for quantification of changes in [^{3}H]DAMGO binding and DAMGO-stimulated [^{35}S]GTPγS binding and for statistical analyses of lesion-induced changes in both. Since grain sizes in the film autoradiographs are larger, coverslip autoradiography was used to assure that coregistration of sections was done accurately, because the emulsion can be apposed tightly to the section and the underlying section is stained with thionin. Thus, the coverslip strategy was used for qualitative assessment of laminar positions of ligand binding and G-protein stimulation. Since the emulsion dries to irregular and unknown thicknesses, this latter technique cannot be used for quantitation.

[^{3}H]DAMGO Autoradiography. Slides were incubated in 50 mM Tris with 1 nM [^{3}H]DAMGO at 25°C for 45 min followed by three buffer washes at 4°C for 1 min each. Nonspecific binding was evaluated in a parallel section coincubated with 1 μM naloxone. Slides were dried and exposed to Hyperfilm for 5 weeks. A subset of slides was apposed to NTB-2 emulsion dipped coverslips for 12 weeks as previously described (Young and Kuhar, 1979; Vogt et al., 1995b). These slides were then developed in Kodak D-19, fixed in Kodak rapid fixer without hardener, and counterstained with thionin.

[^{35}S]GTPγS Autoradiography. [^{35}S]GTPγS autoradiography was performed as previously described (Sim et al.,1996). Slides were brought to room temperature and then equilibrated in 50 mM Tris buffer (pH 7.4) containing 3 mM $MgC1_2$, 0.2 mM EGTA, and 100 mM NaCl (TME + NaCl) for 10 min at 25°C. Sections were then incubated in TME + NaCl with 2 mM GDP for 15 min at 25°C. Sections were then incubated in 10 μM DAMGO, 0.04 nM [^{35}S]GTPγS, and 2 mM GDP in TME + NaCl at 25°C for 2 h. Basal binding was determined in the absence of agonist. Slides were rinsed for 2 min, twice in 50 mM Tris buffer (pH 7.0 at room temperature) at 4°C, then for 30 s in dH_20 at 4°C. Slides were dried overnight and exposed to Reflections film for 48 h. A subset of slides was processed for coverslip autoradiography as described above, with a 2-week exposure time.

Data Analysis. A Sony XC-77 video camera was used to digitize the films, and they were analyzed with NIH Image for Macintosh computers. Values for [^{35}S]GTPγS binding were expressed as nCi[^{35}S]/g of tissue with basal binding subtracted from total binding. [^{14}C] values were corrected for [^{35}S] based on incorporation of [^{35}S] into sections of frozen brain paste (Sim et al., 1996). For [^{3}H] binding, nonspecific binding was subtracted densitometrically from total binding and resulting values are expressed as pCi/mg of tissue. Identification of each layer in anterior and posterior cortex was based on previous cytoarchitectural studies (Vogt and Peters, 1981). Data are reported as mean ± standard error, and statistical significance was determined by two-tailed Student's t tests with an α of 0.05 (JMP software; SAS Institute, Cary, NC). This α was used when paired means were compared for a single area and for those layers in which a specific hypothesis predicted reduced binding for one or two

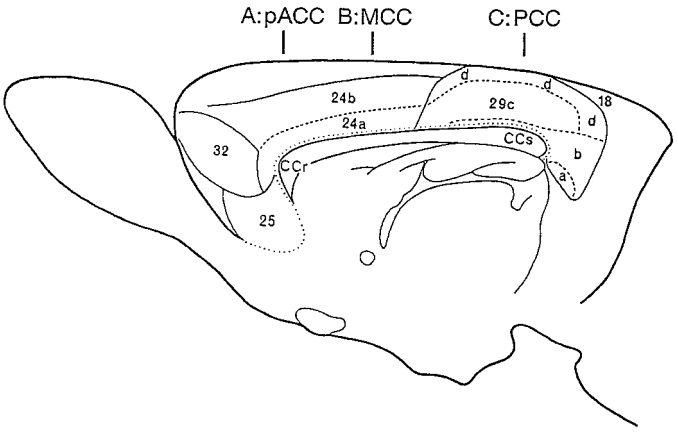

Fig. 1. Topographical distribution of areas in the rat cingulate gyrus. The three rostrocaudal levels from which sections were sampled for the present study are indicated. A, perigenual ACC area 24; B, midcingulate area 24b′; and C, posterior cingulate area 29. CCr, corpus callosum, rostrum; CCs, corpus callosum, splenium.

cortical layers. Because noradrenergic inputs terminate primarily in layer I, a test of the predicted reduction in binding following anti-DBH-saporin lesions was made with an α of 0.05 in layer I for the three areas assessed. In the case of the undercut lesions, only one area was analyzed and the same α was used. This α was Bonferroni corrected for analyzing basal levels of DAMGO-stimulated [^{35}S]GTPγS following anti-DBH-saporin lesions because there were 15 comparisons (five layers for areas 24, 24′, and 29), and no specific hypothesis predicted a change in activity for any layer. One-way ANOVA and Scheffe comparisons were also employed (GB-STAT Software, Silver Spring, MD) to evaluate the density of binding in each layer and between areas.

Films were also analyzed using the receptor binding portion of the Analytical Imaging Station (Imaging Research Inc., St. Catharines, Ontario). This system allows a direct overlay of the Nissl section and the autoradiogram for precise matching of the layers of interest.

Coverslipped autoradiography slides were analyzed qualitatively for the details of laminar binding patterns in relation to thionin-stained neurons to verify findings with film. This tissue was assessed in light of the quantitative findings, and photographs are presented to demonstrate lesion-induced changes in receptor binding and activation of G-proteins.

Results

Laminar Distribution of [^{3}H]DAMGO and DAMGO-Stimulated [^{35}S]GTPγS Binding. Figure 1 is a schematic reconstruction of rat medial cortex (Vogt and Peters, 1981) and shows the three rostrocaudal levels from which sections were sampled. Sampling of each layer was performed with a 38-mm^2 rectangle whose placement on the autoradiograph was guided by adjacent thionin-stained sections. An example of the sampling procedure in area 24b is shown for both [^{3}H]DAMGO and DAMGO-stimulated [^{35}S]GTPγS binding in Fig. 2. Sampling is shown for the nonablated hemisphere in all three sections and the ablated hemisphere for the thionin section only so that reductions in binding can be observed easily in the ablated hemisphere. There is some shrinkage in the ablated hemisphere after removal of afferent axons, and there is a narrow rim of gliosis surrounding the lesion.

The highest overall [^{3}H]DAMGO binding was in perigenual areas 32 and 24. Total binding for all layers in area 24 was 14,806 pCi/mg of tissue, midcingulate area 24′ had 6,020 pCi/mg, and there were 4,082 pCi/mg in posterior area 29. DAMGO-stimulated [^{35}S]GTPγS binding was highest in areas 24 and 24′ with total binding for all layers in area 24 at 1777 nCi/g, area 24′ at 1606 nCi/g, and 780 nCi/g in posterior area 29. Figures 3 and 4 show the laminar distribution of [^{3}H]DAMGO binding and DAMGO-stimulated [^{35}S]GTPγS binding throughout cingulate cortex in control cases. There were some laminar heterogeneities in binding, i.e., peak levels in one or more layers when compared with remaining layers, and these were analyzed with a one-way ANOVA and Scheffe tests.

A one-way ANOVA was performed on the density of binding in each layer of each area. The only significant F occurred with [^{3}H]DAMGO in area 24 ($F = 54$, $p = 0.0001$). A within-group Scheffe comparison confirmed a peak in binding in layer I ($p < 0.01$) with a smaller peak in layer VI ($p < 0.01$) as reported previously (Vogt et al., 1995b). Because DAMGO-stimulated [^{35}S]GTPγS binding is similar in areas 24 and 24′ (Fig. 4B; statistics below), these areas were combined, and a one-way ANOVA was performed ($F = 6.13$, $p = 0.0004$) as

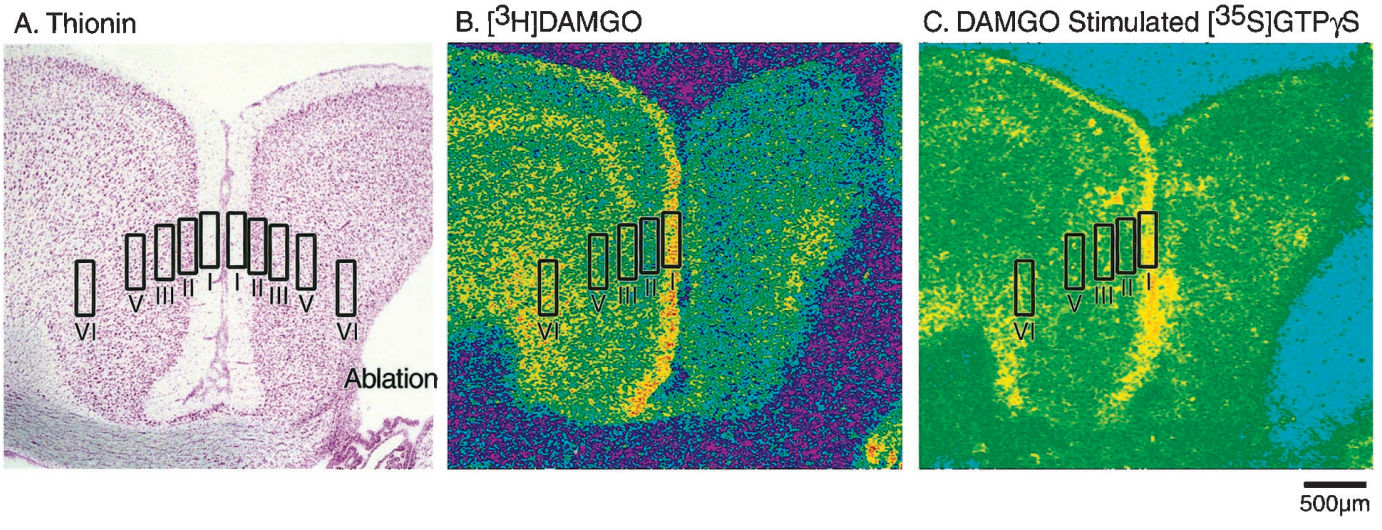

Fig. 2. An example of the sampling procedure for area 24b. The sampling rectangle was placed on each layer in the autoradiographs by guidance for laminar architecture using adjacent thionin-stained sections.

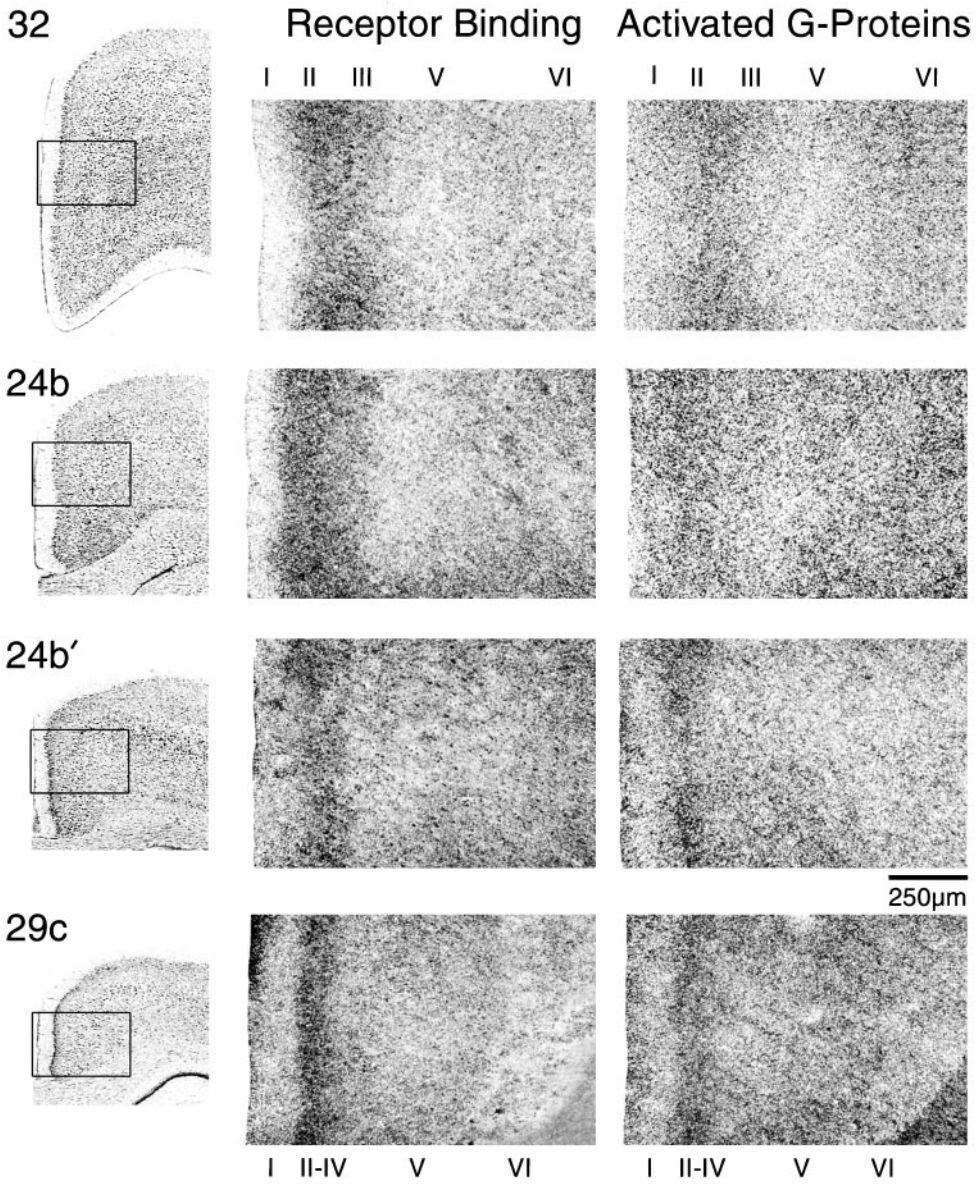

Fig. 3. Photomicrographs of thionin-stained coronal sections throughout cingulate cortex in a control case and darkfield photomicrographs of [^{3}H]DAMGO and DAMGO-stimulated [^{35}S]GTPγS. High binding in layers I and III-V of areas 32 and 24b is apparent. It can also be seen that there is a relatively higher proportion of G-protein activity to μ-receptor binding in areas 24b′ and 29c.

well as a Scheffe comparison. Significant differences were in layers I, II, and V. Protected *t* tests were done to evaluate the difference between areas 24′ and 29 for receptor binding and areas 24 and 24′ for stimulated G-protein binding, and no significant differences were found. Therefore, these groups were combined. When areas 24′ and 29 were combined for receptor binding and compared with area 24, there were significant differences in all layers ($p < 0.0001$). DAMGO-stimulated [^{35}S]GTPγS binding also showed significant differences in all layers when areas 24 and 24′ were combined ($p < 0.05$). Because area 24′ mirrored that in area 24 in stimulated G-protein binding, whereas it mirrored area 29 in receptor binding, midcingulate area 24′ has a unique μ-opioid architecture in comparison to areas 24 and 29.

Receptor Binding and Receptor-Activated G-Proteins after Undercut Lesions. Undercut lesions remove all afferent axons to ACC, while leaving cortical neurons intact (Vogt, 1993). Thionin-stained sections confirmed that these lesions were limited to the white matter without damaging adjacent cingulate cortex. Because the knife passed through posterior cingulate cortex to assure removal of rostrally directed fibers in the cortex, only areas 32 and 24 were analyzed. There was a significant decrease in [^{3}H]DAMGO binding in all layers of areas 32 and 24 following the lesions. The largest reduction occurred in layer I (65%, $p = 0.009$), and the smallest decrease was in layer II (28%, $p = 0.01$; Fig. 5A). The binding was homogeneous across all layers following the lesion.

In contrast to receptor binding, reductions in DAMGO-stimulated [^{35}S]GTPγS binding in ACC occurred in three layers (Fig. 5B): layer I (59%, $p = 0.01$), layer II (64%, $p = 0.007$), and layer III (64%, $p = 0.02$). The overall laminar binding pattern, including peaks in layers I and VI, remained following the lesions. Thus, the changes in receptor binding and activated G-proteins after undercut lesions were not equivalent in ACC.

Because lesions could change spontaneous receptor activation of G-proteins, they may alter basal activity, which is subtracted from total activity in the above calculations. To assure that changes in DAMGO-stimulated [^{35}S]GTPγS were

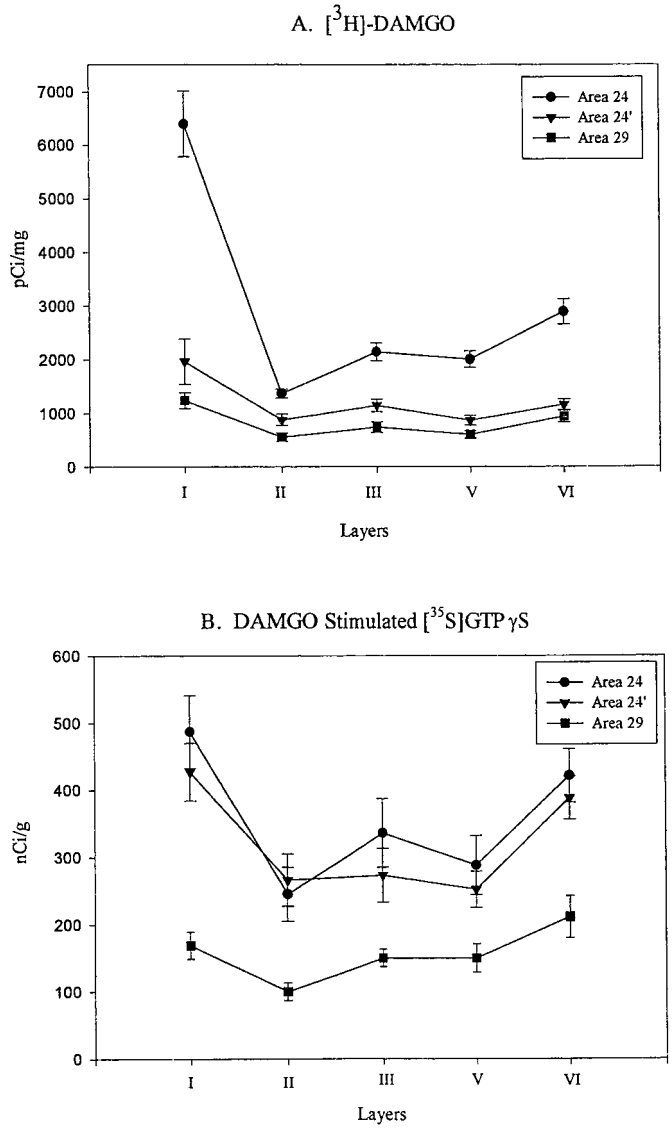

Fig. 4. Laminar distribution of [^{3}H]DAMGO (A) and DAMGO-stimulated [^{35}S]GTPγS (B) binding quantified in control cases in areas 24, 24′, and 29.

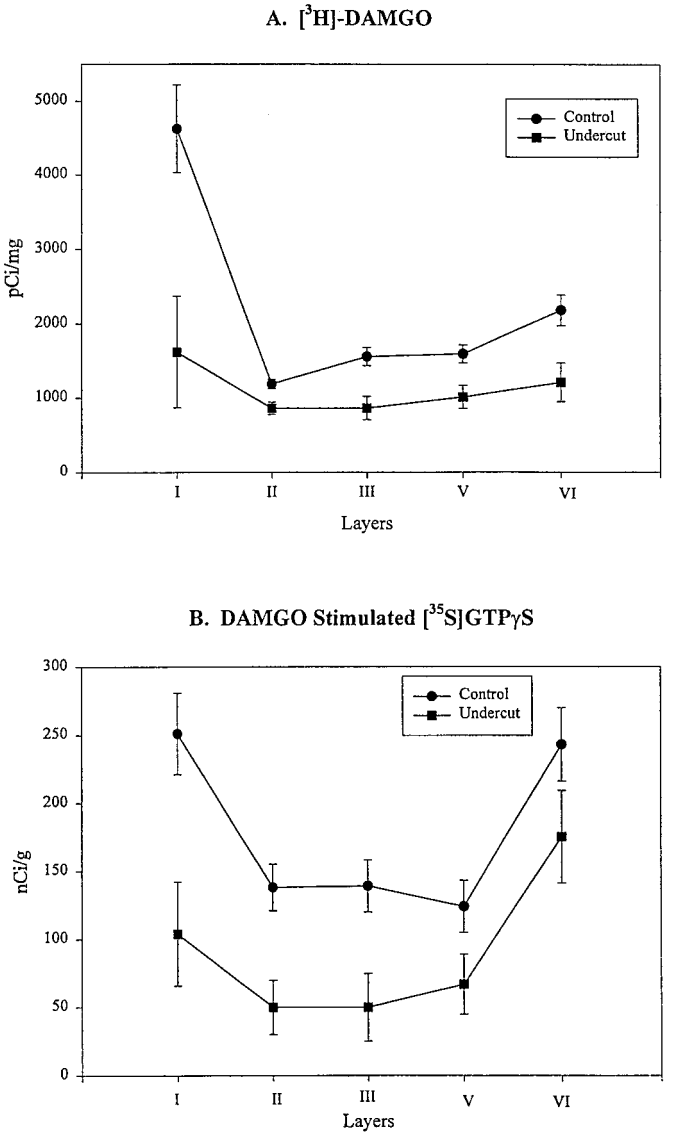

Fig. 5. Binding of [^{3}H]DAMGO (A) and DAMGO-stimulated [^{35}S]GTPγS (B) in area 24 in controls and cases with undercut lesions. Most prominent changes for DAMGO binding were in layers I and VI, whereas G-protein activity was reduced mainly in layers I–III.

not the result of changes in basal levels of activity, basal activity was evaluated for control and undercut hemispheres. Undercut lesions were not associated with changes in basal levels of activity in layers I, III, and V. In layers II and VI, there were significant increases in basal stimulation. This difference, however, would have amplified further the significant reduction in layer II activity reported above. Because basal activity in layer VI increased after the lesion, the nonsignificant reduction in stimulated activity reported above could actually be larger. Thus, changes in basal activity associated with the lesions did not account for most DAMGO-stimulated [^{35}S]GTPγS changes.

Binding after Anti-DBH-Saporin Injections. Figures 6A and 7A show the laminar distribution of [^{3}H]DAMGO binding in perigenual area 24. Because the anti-DBH-saporin injections were placed in the left lateral ventricle and damaged ipsilateral cortex, measurements were made only in the contralateral hemisphere. There was a 31% decrease in [^{3}H]DAMGO binding in layer I of area 24 ($p = 0.05$), and no other changes in binding in areas 24, 24′, or 29 (Fig. 6, A–C). This decrease in binding fulfilled the hypothesis enumerated earlier in relation to noradrenergic inputs to layer I of area 24.

DAMGO-stimulated [^{35}S]GTPγS binding increased in layer II of area 24 (Figs. 6D and 7B) by 36% ($p = 0.03$) and in layer I of area 29 by 29% ($p = 0.03$) (Fig. 6F). Because there were not specific hypotheses to guide this statistical analysis, Bonferroni correction was made of the α for 15 comparisons; i.e., five layers for areas 24, 24′, and 29. This correction meant that no changes were statistically significant. Finally, basal levels of DAMGO-stimulated [^{35}S]GTPγS were assessed following these lesions in anterior, middle, and posterior cortices. After a Bonferroni correction for 15 comparisons, there were no significant changes in basal levels of activity.

Correlation Analysis. The assay conditions for homogenized tissue (Maher et al., 2000) cannot be applied to autoradiography because agonists were used at a single equilib-

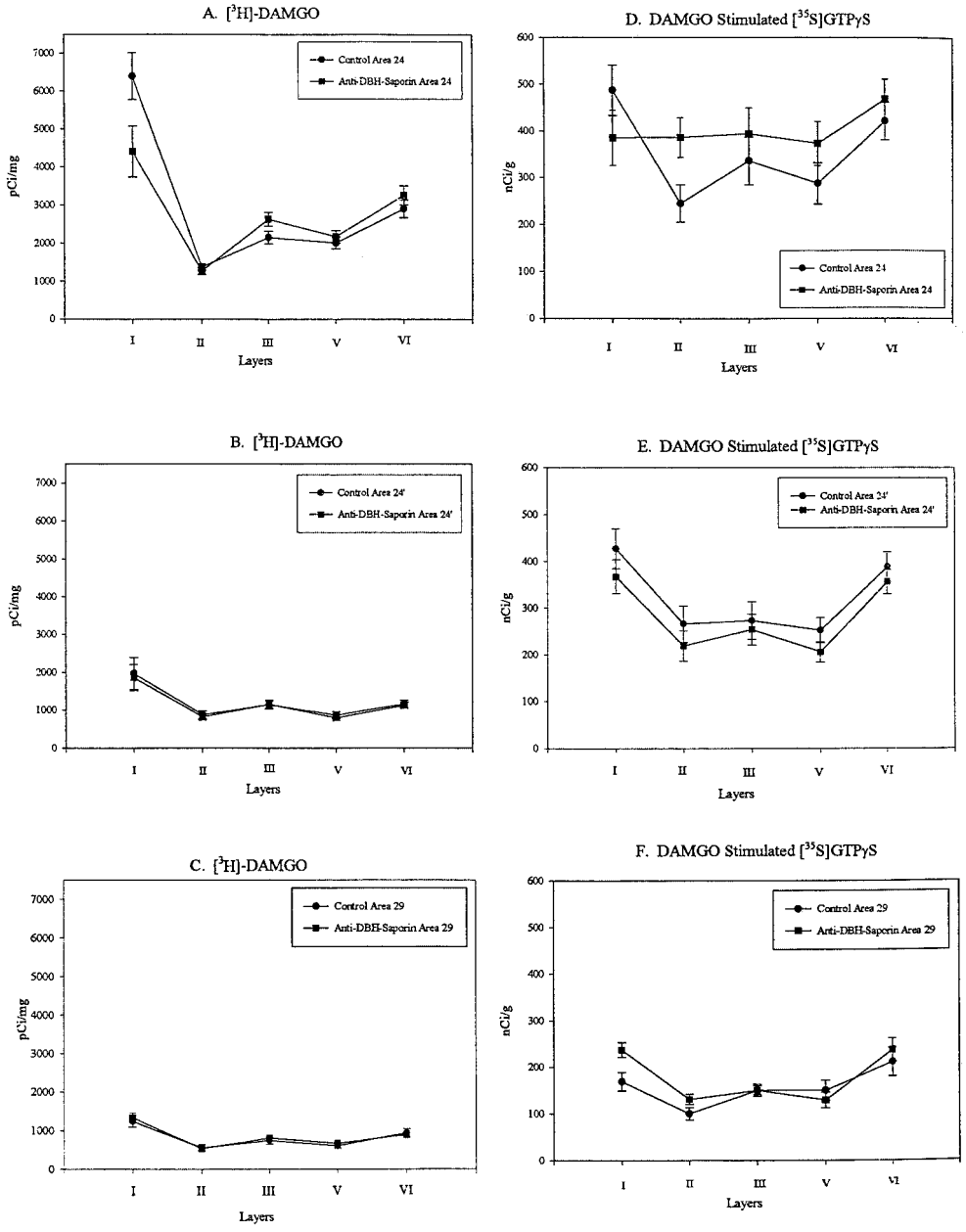

Fig. 6. Laminar distributions of [^{3}H]DAMGO binding in areas 24, 24′, and 29 in controls and cases with anti-DBH-saporin lesions (A–C) and DAMGO-stimulated [^{35}S]GTPγS binding in the same cases (D–F). The only change in [^{3}H]DAMGO binding can be seen in layer I of area 24, whereas there is an increase in DAMGO-stimulated [^{35}S]GTPγS binding in layer II of area 24 and layer I of area 29.

rium concentration. Instead, a correlation analysis was used to relate DAMGO binding and GTPγS-stimulated binding. Plots of [^{3}H]DAMGO binding versus DAMGO-stimulated [^{35}S]GTPγS for each layer of an area in each of the three primary divisions of cingulate cortex are shown in Fig. 8. In each area the points for layer I stand out as potential outliers and the undercut lesions (inverted triangles), which remove a large proportion of DAMGO binding, reduced overall layer I binding to a level that is similar to those in the underlying cellular layers. Furthermore, although a significant one-way ANOVA was present when the layer I values were included, removal of layer I improved the F statistic from 0.70 to 3.6 times larger as follows: area 24b, $F = 33$ with layer I and 56 without; area 24b′, $F = 76$ with layer I and 277 without; and area 29c, $F = 93$ with layer I and 178 without. Thus, the layer I values were treated as outliers, and the linear regressions were calculated for the cellular layers only. The correlation coefficients were 0.87 for area 24b, 0.70 for area 24b′, and 0.84 for area 29c.

Assessment of the graphs suggests that layer I in all areas has a generally greater proportion of MOR to μ-activated G-proteins than is true for the underlying cellular layers. In the cellular layers, as noted above, there is a high correlation between [^{3}H]DAMGO binding and DAMGO-stimulated [^{35}S]GTPγS binding. Finally, the anterior areas have a broader range of G-protein activity than is true for posterior area 29. In light of the changes following both deafferentation lesions and alterations in layer I, it is possible that axon terminals have a higher ratio of μ-binding sites to μ-activated G-proteins than for neuronal somata and proximal dendrites.

Discussion

Perigenual area 24 had the highest overall [^{3}H]DAMGO binding with a peak in layer I, whereas area 24′ and posterior cingulate cortex area 29 had lower overall binding and a modest elevation in layer I. In contrast, DAMGO-stimulated

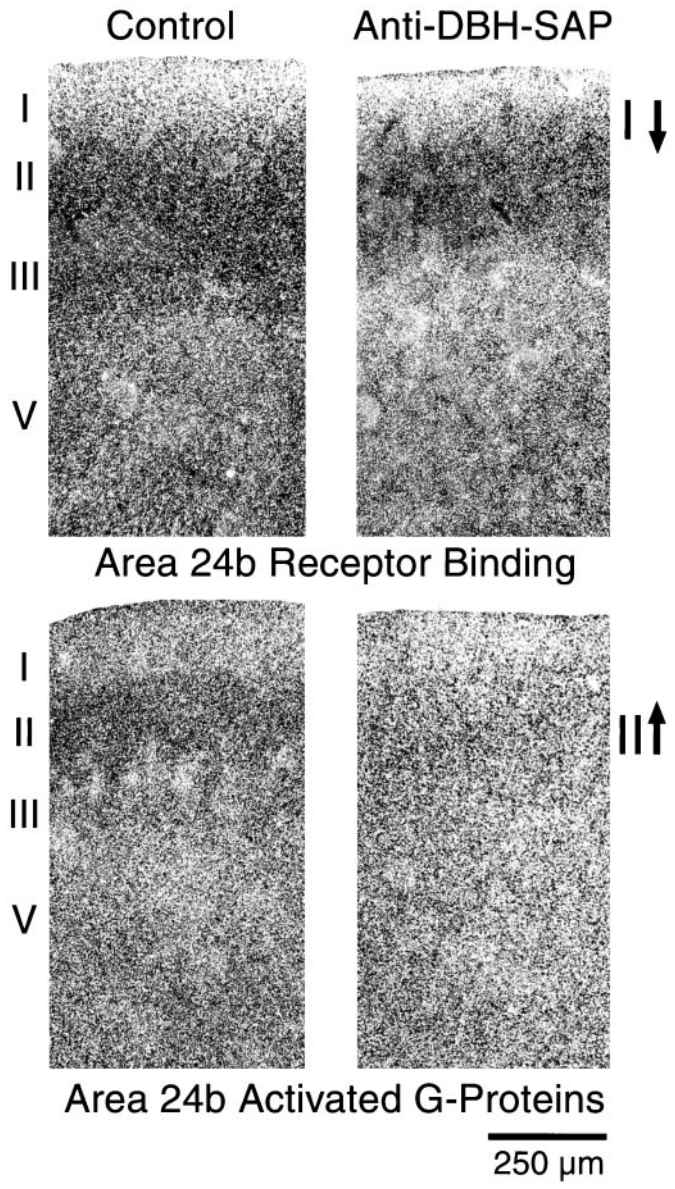

Fig. 7. Darkfield photomicrographs of [^{3}H]DAMGO and DAMGO-stimulated [^{35}S]GTPγS binding in a control case and a case with an anti-DBH-saporin lesion. The changes in binding in layers I and II following the lesions are noted with arrows in sections from the hemisphere contralateral to the one with the cannula penetration.

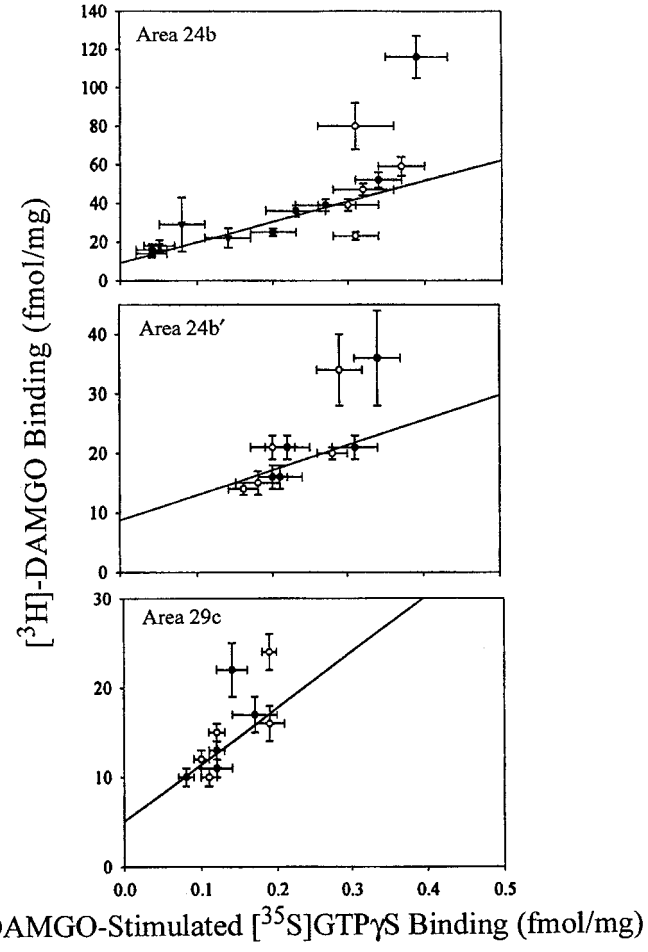

Fig. 8. Correlation between the levels of [^{3}H]DAMGO and DAMGO-stimulated [^{35}S]GTPγS binding in areas 24b, 24b′, and 29 in controls (●), undercut lesions (▼), and anti-DBH-saporin (○) lesions. Values for layer I in all areas were outliers as discussed in the text and linear regressions were performed for the cellular layers only.

[^{35}S]GTPγS binding in area 24′ mirrored that in area 24 rather than area 29, suggesting area 24′ has a unique μ-opioid architecture. Thus, the proposal that ACC is comprised of perigenual and midcingulate divisions based on circuit and functional observations is supported. The presence of a unique opioid architecture in these regions is probably caused by a more robust midline and intralaminar thalamic projection to layer I in perigenual cortex (Herkenham, 1979) and expression of MOR on these terminals (Vogt et al., 1995b) and in the thalamus (Vogt et al., 1992). There may also be a higher level of MOR expression by somatodendritic structures in perigenual cortex than is the case for posterior cortex. The net result of such an organization is that perigenual cortex is under tighter opioid regulation than middle and posterior cingulate cortices.

Area 24 undercut lesions reduced [^{3}H]DAMGO binding in all layers with the greatest reduction in layer I, whereas [^{35}S]GTPγS binding was only reduced in layers I to III. Anti-DBH-saporin injections reduced [^{3}H]DAMGO binding in layer I of area 24 as predicted, but there were no changes in areas 24′ or area 29. Lesion-induced DAMGO-stimulated [^{35}S]GTPγS binding did not correspond to changes in receptor binding. There was evidence that [^{35}S]GTPγS increased in layer II of area 24 and decreased in layer I of area 29 with no changes in area 24′. The lack of a close correspondence between receptor and G-protein regulation has been noted after chronic heroin self-administration (Sim-Selley et al., 2000). In this latter condition, MOR binding increased, while μ-agonist-stimulated GTPγS binding declined after chronic heroin treatment. Moreover, previous studies comparing the regional distribution of [^{3}H]naloxone binding with DAMGO-stimulated [^{35}S]GTPγS binding revealed that this relationship was not the same in each region of the telencephalon, with the amplification factors of μ-activated G-proteins to μ-receptor binding varying from 8 to 40 (Maher et al., 2000).

The cause of this complex relationship is not clear, but several possible explanations exist. First, it is known that μ-receptors catalytically activate multiple G-proteins of different types, with more than one G-protein activated per receptor (Chakrabarti et al., 1995). Thus, under these condi-

tions a change in receptor binding may not reflect a similar change in receptor-activated G-proteins. Second, it is possible that a significant number of μ-receptors detected by [^{3}H]DAMGO binding in the present study may not be coupled functionally. Indeed, at the level of cellular resolution provided by autoradiography, some of these receptor binding sites may be sequestered in intracellular sites and therefore not available for signal transduction. Finally, it is important to note that changes in [^{3}H]DAMGO binding may not reflect changes in μ-receptor number. Because DAMGO is a μ-agonist and binds to different affinity states of μ-receptors (Childers and Snyder, 1979), it is possible that changes in [^{3}H]DAMGO binding reflect changes in the proportion of high- and low-affinity agonist sites. If so, such changes would result in complex relationships between receptor binding and μ-activated G-proteins.

In view of the demonstration of MOR and a proportionately high level of coupled G-proteins on axon terminals derived from the locus coeruleus, it must be considered that opioid systems not only regulate acute pain processing but also can modify stress responses in ACC. Indeed, an established model of stress employs noxious stimulation with intermittent and unavoidable electrical foot shock (Imaki et al., 1993), and this model activates neurons in the locus coeruleus (Li and Sawchenko, 1998). Because stress is a consequence of noxious stimulation, one may predict a close link between cortical areas regulating both functions, however, this is not the case. Thirty minutes of intermittent electrical footshock in rat elevates c-fos activity mainly in areas 25 and 32 but not cortex dorsal to the corpus callosum (Li and Sawchenko, 1998). Studies of nociceptive neurons in rat and rabbit show that nociceptive neurons are primarily in dorsal area 24b (Sikes and Vogt, 1992; Hsu and Shyu, 1997), suggesting that the closest association of acute stress and pain is with the affective component of each in areas 25 and 32, but not the dorsal area 24b and adjacent area 8. Thus, MOR-mediated reductions in noradrenaline release in area 24b could result in a reduction in a stress response in dorsal cingulate cortex during processing of pain information. Although ACC has a high level of opioid receptor binding (Vogt et al., 1995a), intrathecal morphine alone or in combination with α-agonists provides effective relief of pain (Yaksh, 1999). Are there chronic pain states in which systemic administration of one or both of these compounds should be employed to target ACC? Perigenual cingulotomy (Brown and Lighthill, 1968) or mid-cingulotomy (Ballantine et al., 1975) is effective in relieving psychiatric symptoms such as major depression, anxiety, aggression, fear, and paranoia. Because morphine alters mood and affective pain ratings (Kupers et al., 1991), it is appropriate to consider morphine for relief of depression, anxiety, and/or aggression when associated with chronic pain. Moreover, because the present study has shown that opioid receptors are expressed on axonal projections of the locus coeruleus in ACC in addition to the locus coeruleus itself, these receptors provide a means of blocking stress associated with chronic noxious stimuli like that produced by cancer and central pain syndromes. Thus, in instances where chronic pain is associated with psychiatric symptoms, reducing ACC activity with a combination of μ-opiate and α-agonists provides an additional benefit to treatments that target spinal or peripheral nociceptive processing.

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