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# Localization of Mu and Delta Opioid Receptors to Anterior Cingulate Afferents and Projection Neurons and Input/Output Model of Mu Regulation

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**Anterior cingulate cortex (ACC) has one of the highest densities of opioid receptors in the CNS and it has been implicated in acute and chronic pain responses. Little is known, however, about which neurons express opioid receptors in their dendrites and axon terminals. The present studies employed experimental techniques to remove afferent axons or classes of projection neurons from rat ACC area 24 followed by coverslip autoradiography to localize changes in binding of [$^3$H]Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) to mu receptors and 2-[$^3$H]D-penicillamine-5-D-penicillamine–enkephalin (DPDPE) to delta receptors. Removal of all afferents to area 24 with undercut lesions did not alter DPDPE binding, but significantly reduced binding of DAMGO in layers I, III, and V. In contrast, removal of all cortical neurons with the excitotoxin ibotenic acid almost abolished DPDPE binding in all layers. The same lesions reduced DAMGO binding in most layers; however, there was a postlesion bimodal distribution in binding with high levels of binding in layer I and moderate levels in layer VI. These data suggest that delta receptors are expressed by cortical neurons, while mu receptors are expressed by both cortical neurons and afferent axons. To explore the distribution of postsynaptic receptors, immunotoxin lesions were made in area 24 by injection of OX7–saporin into the caudate and/or thalamic nuclei. Almost complete removal of projection neurons to these targets in layers Vb and VIa did not alter DPDPE binding, while the lesions reduced DAMGO binding in all but layer II. Removal of layer Vb corticostriatal projection neurons with caudate OX7–saporin injections reduced binding only in this layer. It is proposed that opioidergic circuits in area 24 are organized according to an input/output model for mu opioid regulation. In this model mu receptors regulate axon terminal activity from the thalamus in layer Ia and the locus coeruleus in layers Ic and II, whereas cortical outputs to the thalamus are modulated via postsynaptic receptors expressed in all layers by thalamocortical projection neurons with somata in layer VI. These opioidergic circuits in ACC are of particular importance because they may regulate responses to chronic nociceptive activity and associated pain perceptions.** © 1995 Academic Press, Inc.

## INTRODUCTION

Positron emission tomography (PET) studies (5, 19, 39) have shown that human anterior cingulate cortex (ACC) is activated by acute, noxious–thermal stimuli. Neurons in rabbit ACC area 24b are nociceptive and have robust responses to noxious mechanical and/or thermal stimuli and almost no somatotopic organization in receptive field coding (38). Direct responses to noxious stimuli may explain why destruction of ACC and/or its underlying white matter (i.e., cingulum bundle) in chronic pain patients alleviates the affective components of responses to noxious stimuli in human (3, 13) and why injections of lidocaine into the cingulum bundle alleviates chronic pain in rat (41).

The contributions of ACC to pain responses may be partially accounted for by its afferent connections from the medial thalamic nuclei and projections to the periaqueductal gray. Lidocaine injections into the medial thalamus disrupt nociceptive activity in area 24, suggesting that this may be the origin of nociceptive activity in ACC (38). In terms of its efferent projections, electrical or chemical stimulation of ACC and adjacent prefrontal cortex inhibits nociceptive neuronal activity in the periaqueductal gray (40). Thus, ACC receives nociceptive inputs and regulates responses to noxious stimuli.

Modulating responses to noxious stimuli may be one function of the widely distributed opioid receptor subtypes in the cerebral cortex. Several lines of evidence suggest that there are intrinsic opioid circuits in ACC. Hiller *et al.* (18) first reported that ACC has one of the highest levels of opiate ligand binding in the CNS. *In vivo* studies using PET confirm the high levels of opiate binding sites in human ACC (21, 35, 50) and that morphine elevates blood flow in a rostral and ventral



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part of cingulate cortex (26). Most opioid binding in this region is to mu and delta opioid receptors, although there is limited binding of kappa ligands, and binding to mu and delta opioid receptors in posterior cingulate cortex is less than half of that in the anterior region (27, 29). Laminar analysis of binding to mu receptors in ACC shows that it is highest in layers I, III, and V (27, 29).

Layer I is a major site of thalamic afferents, and thalamic lesions show that these neurons express presynaptic mu opioid receptors in layer I of rat posterior cingulate cortex (46). These mu receptors may be synthesized in the laterodorsal nucleus (10, 27) because the ventral part of this nucleus projects to posterior cingulate cortex in rabbit (51). Since thalamic neurons that project to the cortex are not opioidergic (12, 16), such presynaptic receptors are heteroreceptors on glutamatergic thalamocortical afferents. Expression of mRNA for mu opioid receptors have shown that, in addition to the midline thalamic nuclei which synthesize these receptors, neurons in the locus coeruleus also synthesize them (10, 26). Since noradrenergic axon terminals occur in ACC (30), there are two extrinsic inputs to ACC that may express mu opioid receptors.

The source of opioidergic control for superficial and deep layers of ACC is via intrinsic circuits. Enkephalin-immunoreactive neurons are in layers II–III and V (23), and those in layer V of ACC form appositions to the somata of large pyramidal neurons (36). Area 24 also has a diffuse (i.e., not layer specific) population of dynorphin B-immunoreactive neurons (12), and a study of mRNA levels for preproenkephalin shows a diffuse distribution of neurons expressing this message throughout cingulate cortex (16). It is important to note, in regard to the wide distribution of opioid receptors and opioidergic neurons throughout the cerebral cortex, that intrinsic opioidergic systems are likely engaged in modulating many responses other than those associated with nociception. In light of the nociceptive properties of ACC, however, the roles of opioid systems in this region of the brain merit consideration in terms of their modulation of responses to acute and chronic noxious stimuli and perceptual and affective events associated with pain.

The present investigation sought to determine the anatomical localization of mu and delta opioid receptors in each layer of area 24 in ACC. The experiments were designed to test the hypothesis that a large percentage of these receptors in deep layers was expressed postsynaptically by cingulate projection neurons. This was demonstrated with immunotoxin ablation techniques combined with autoradiographic ligand binding to localize mu and delta opioid receptors to cortical sublayers. Since neurons in two brainstem nuclei which project to ACC synthesize mu opioid receptors (i.e., midline/intralaminar thalamic and locus coeruleus nuclei), deafferentation lesions were placed in order to estimate the density of presynaptic receptors in each layer of ACC. Finally, since the contributions of opioid receptors to cortical function are poorly understood, a model of ACC circuitry is proposed to identify critical points at which mu opioid receptors modulate afferent axon terminals and corticothalamic projection neurons.

## MATERIALS AND METHODS

### *Lesion Strategy*

Experimental techniques were employed to localize opioid receptor binding to afferent axons and cortical neurons. The ACC was deafferented of all extrinsic inputs with undercut lesions. Cingulate cortex in the rat is particularly amenable to such an operation because it is oriented on the medial surface such that a scalpel blade can be drawn beneath the cortex along its full rostrocaudal extent. With additional cuts in the rostral and caudal white matter to ensure complete disruption of its inputs, postlesion binding was associated with only local intracortical elements. A schematic diagram of this outcome is shown in Fig. 1. In contrast, injections of the excitotoxin ibotenic acid into area 24 destroy all neurons and their associated dendrites and transmitter receptors, while sparing afferent axons and supporting elements (37, 44). The undercut and neurotoxin lesions have been used previously to localize muscarinic receptors (43, 45).

Immunotoxin lesions, such as those made with OX7–saporin, provide a means of removing specific populations of cortical projection neurons through their suicide transport of the toxin. As shown in Fig. 1, injection of the toxin into a cortical projection site such as the caudate nucleus destroys only pyramidal neurons which project to that nucleus. Immunotoxins are antibodies to particular cellular molecules that are covalently linked to cytotoxins that exert their action within the cell. OX7–saporin is composed of an antibody to the abundant membrane glycoprotein Thy 1.1 covalently bound to the endonuclease saporin. When injected into the brain, the OX7 antibody binds to the cell surface, is endocytosed, and destroys neurons through saporin RNase activity (53). Injections into the CNS lead to the axonal endocytosis of OX7–saporin, retrograde transport to the neuronal somata, and neuronal destruction via a process termed "suicide transport" (52). Crino *et al.* (8) used this technique to localize postsynaptic serotonin receptors by placing immunotoxins in thalamic and caudate nuclei to destroy cingulate layer VI and layer V neurons, respectively, followed by coverslip autoradiography.

### *Surgical Procedures*

Male Long–Evans rats (350–400 g) were anesthetized with Chloropent (0.3 ml/100 g body wt. ip) and one of the following lesions was placed: (1) Unilateral

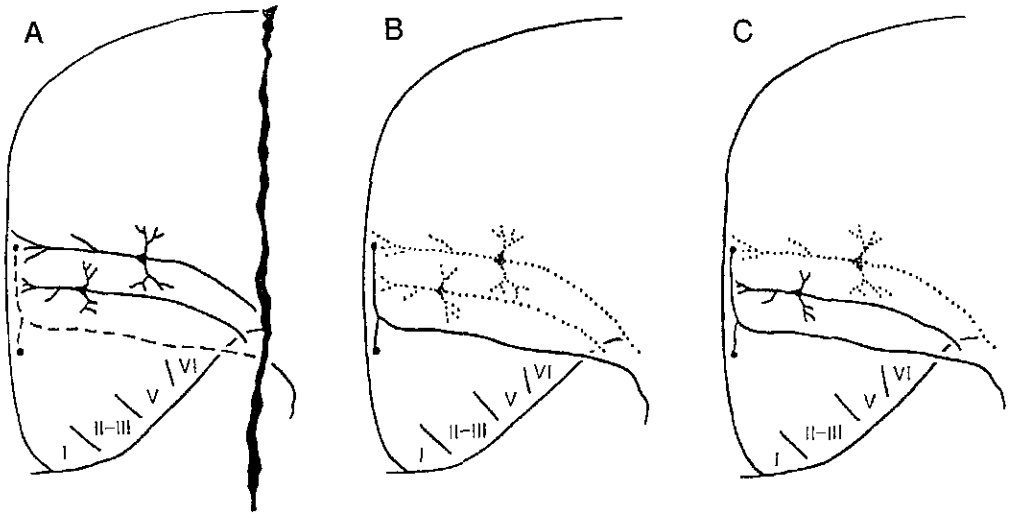


**FIG. 1.** Schematic summary of the experimental consequences of various lesions. (A) Undercut lesions remove all cingulate cortical afferents, while leaving cortical neurons intact. (B) Excitotoxin lesions destroy all cortical neurons but spare afferent axons. (C) Immunotoxin lesions destroy specific classes of cortical projection neurons, whereas other cortical neurons and afferent axons remain intact.

undercut lesions were made with a scalpel blade that was passed 1.0 mm lateral to the midline, 3.5 mm ventral to the cortical surface extending from 2.0 mm rostral to bregma to 5.5 mm posterior to bregma. Coronal knife cuts were made at the rostral and caudal limits of the knife cut 1.0 to 1.5 lateral to the midline to ensure complete deafferentation ($N = 6$). (2) Injections of the excitotoxin ibotenic acid (1–2 µl per injection of 10 µg/µl 0.9% saline) were placed unilaterally into area 24 above the genu of the corpus callosum ($N = 4$). (3) Unilateral injections of OX7–saporin (1.25–2.25 µg/0.5–1.0 µl of phosphate buffer) were made into different parts of the caudate nucleus (stereotaxic coordinates of +0.7 mm anterior to bregma, 2.2 mm lateral to the midline, and 3.7–4.5 mm ventral to the cortical surface; $N = 21$). We thank Professor F. Stirpe for the generous supply of OX7–saporin for these injections. After a 2-week postoperative survival period, animals were sacrificed with $CO_2$ and perfused intracardially with 50 ml cold Krebs–Henseleit buffer, pH 7.4. Brains were removed, rapidly frozen in hexane (−75°C) for 2 min, and stored at −80°C.

*Tissue Preparation, Incubation, and Autoradiography*

Coronal, 16-µm-thick sections were cut at −21°C and thaw mounted on chrome–alum-coated microscope slides. Alternate sections were cut at three times this thickness for thionin staining to verify the location and extent of each lesion. To determine 2-[$^3$H]D-penicillamine–5-D-penicillamine–enkephalin (DPDPE) binding, sections were preincubated in 50 m*M* Tris with 5 m*M* $MgCl_2$, 2 mg/ml bovine serum albumin, 20 µg/ml bacitracin, 100 m*M* NaCl, and 50 µ*M* GTP at 25°C for 15 min. The sections were then washed twice in a similar buffer to remove the GTP at 25°C for 5 min each. Incubation of [$^3$H]DPDPE (sp act 43 Ci/m*M*; New England Nuclear) was conducted in 50 m*M* Tris with 2 mg/ml bovine serum albumin at 25°C for 60 min followed by three buffer washes at 4°C for 10 min each. Nonspecific binding was assessed in a parallel series of sections coincubated with 1 µ*M* DPDPE. To determine [$^3$H]Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) binding, sections were incubated in 50 m*M* Tris with 1 n*M* [$^3$H]DAMGO (sp act 30.3 Ci/m*M*; New England Nuclear) 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 µ*M* levallorphan kindly provided by Hoffmann–LaRoche, Inc.

Coverslip autoradiographs were prepared as in previous studies (43, 45). Coverslips were acid cleaned and dipped in Kodak NTB-2 nuclear tract emulsion. The dried coverslips were attached to slides with cyanoacrylate and exposed in the dark at −20°C for 3 months. All of the autoradiographs were developed in Kodak D-19 without hardener, fixed in Kodak Rapid Fixer, and counterstained with thionin. In order to ensure that the autoradiographs were developed in the linear range of the emulsion, test series were developed at different exposure times (i.e., 1, 2, or 3 months) and trial slides developed for different times and temperatures in the Kodak D-19. The final parameters were set such that grain densities in the layer with highest binding (i.e., layer Ia) were optimal without producing overlap in grains by more than 10% of the total grain density. This also ensured that the automatic grain counter could efficiently analyze grain densities with a minimum correction for overlapping grains as discussed below.

*Lesion Assessment and Quantification of Ligand Binding*

Each case was initially assessed for the extent of the lesion using the thick, thionin-stained sections. Area 24

was defined as an agranular cortex in the rostral cingulate region (44). Drawings of the lesions in cortical layers or subcortical nuclei were made with either a drawing tube attachment to the microscope or an Aus Jena projector. Undercut lesions were used if the scalpel blade did not injure the deep layers of area 24. Successful ibotenic acid lesions resulted in a complete removal of cortical neurons, in which instance the laminar architecture was not available for evaluating binding. The determination of laminar binding patterns was accomplished by referring to intact parts of area 24b dorsal to the injection site (i.e., area 24 was never completely removed). Binding was quantified in the center of each lesion, not at its periphery, where intact dendrites may occur.

Although OX7–saporin injections were made into the caudate nucleus and many were restricted therein, other cases had different sizes of injections with varying amounts of spread caudal into the thalamus. Thus, cases were classified either as pure caudate injections in which neuron loss and gliosis were limited to the caudate nucleus or as caudate and thalamic nuclei injections when spread beyond the caudate involved the posterior thalamus to involve the mediodorsal, centrolateral, and parafascicular nuclei. Cases that involved only the anterior thalamic nuclei were not used because of the limited projections of the anteromedial nucleus to anterior cingulate cortex. Removal of only one or two layers of neurons did not result in shrinkage of cortical layers. Examples of laminar thicknesses following caudate and thalamic injections include the following for normal/ablated hemispheres: I, 202 ± 8/203 ± 10 mm; Va, 277 ± 11/239 ± 15 mm; and VIa, 387 ± 25/325 ± 28 mm. Injections of the caudate nucleus resulted in laminar thicknesses as follows: I, 173 ± 8/160 ± 6 mm; Va, 239 ± 28/243 ± 18 mm; and VIa, 313 ± 26/332 ± 27 mm.

The extent of cortical neuron degeneration was evaluated in drawings of the perikarya of neurons throughout area 24. The perikarya of neurons were drawn with a drawing tube attachment to the microscope in 160-μm-wide strips of area 24b and neurons counted in each layer. Most of the neurons in layer VIa were lost when the posterior thalamus was involved in the injection, while many but not all of the neurons in layer Vb were removed following the caudate nucleus injections. Examples of perikaryal drawings from similar cases have been published (8). Since corticothalamic neurons project their axons through the caudate nucleus, injections limited to the caudate nucleus destroyed a small number of layer VI neurons as reported under Results.

Grain densities were quantified with a computerized image analysis sytem (Image Technology Model 1000, Donsanto Corp., Natick, MA). Grains in the emulsion were viewed with darkfield microscopy and counted per 2500 $\mu m^2$ of a cortical layer and then corrected visually for overlapping grains that the machine incorrectly counted as a single grain. Specific binding was determined bilaterally in layers Ia, Ib, Ic, II, III, Va, Vb, VIa, and VIb in two or three sections per brain by subtracting grain number in sections coincubated with unlabeled blockers from numbers in sections that were not coincubated in the blocker. The mean ± SEM was calculated for each layer in each hemisphere by lesion group. When comparing the differences between mean grain densities on control and contralateral, ablated hemispheres, two-tailed $t$ tests were used and $P < 0.05$ was accepted as significant.

## RESULTS

### *Laminar Distribution of DPDPE and DAMGO Binding*

The solid lines in Fig. 2 are for control levels of specific binding of DPDPE to the delta and DAMGO binding to mu opioid receptors. Specific DPDPE binding in area 24 is at peak levels in layers V and VIa, whereas binding in layers I–III and VIb are lower in density. Specific binding of DAMGO in area 24 is highest in layers Ia and Ib and significantly lower in all other layers.

### *Binding Following Deafferentation Lesions*

Complete removal of afferent axons with undercut lesions did not alter DPDPE binding in area 24 (Fig. 2). Although there was no change in DAMGO binding in layers II and VI following the same lesions, there were significant reductions in layers I, III, and V. Binding of DAMGO in layer I was reduced by larger percentages (49–59%) than in layers III (45%), Va (40%), and Vb (29%).

### *Binding Following Excitotoxin Lesions*

The binding of DPDPE is almost completely abolished by ibotenic acid lesions (Fig. 2). Specific binding of DAMGO in the same cases is reduced, but in a different pattern. Although ibotenic acid lesions significantly reduced DAMGO binding in all layers, a high level of binding remained in layers Ia and Ib. This binding was 80 and 72%, respectively, of control specific binding in these sublayers. Figure 3 shows an autoradiograph of the border of an ibotenic acid lesion in area 24. Notice that DAMGO binding in unablated cortex has a pattern similar to that in control cases, including lowest binding in layer II. In the ibotenic acid ablated cortex, DAMGO binding in layers Ia and Ib is quite high, although at a reduced level to that which occurs in the unablated cortex. It also should be noted in Fig. 2 that, although DAMGO binding was significantly reduced in layers V and VI, there was a gradual elevation in binding that reaches a deep-layer peak in layer VIb.

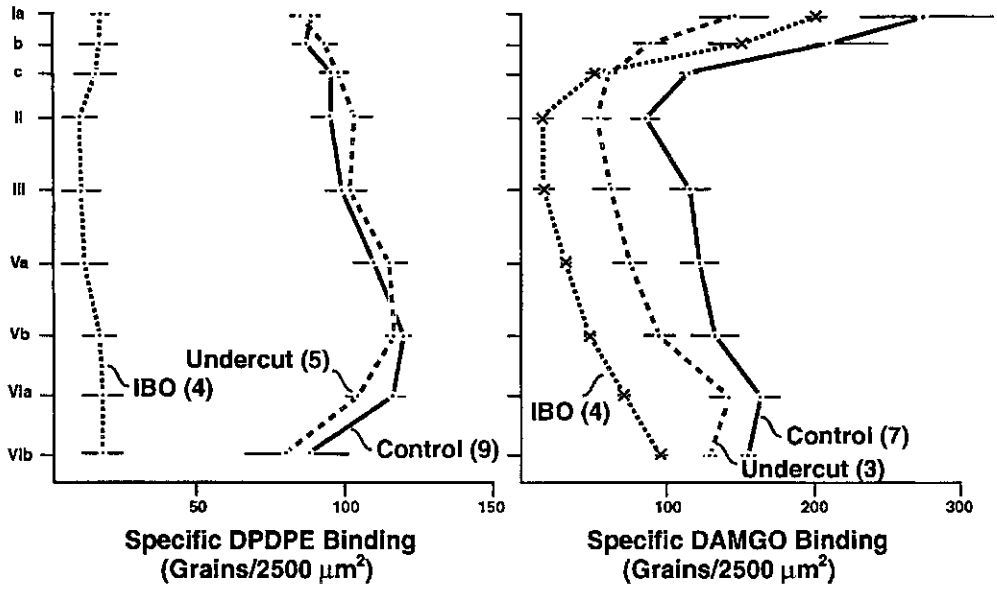


**FIG. 2.** Ligand binding in area 24 in control cases (solid lines; numbers in parentheses are the number of cases in each group) and in undercut and ibotenic acid-ablated cases. Specific binding in this and subsequent figures is the mean ± SEM for each layer.

Thus, postibotenic–acid–lesion binding of DAMGO had a pronounced bilaminar distribution.

*Binding Following Immunotoxin Lesions*

Two groups of OX7–saporin injections were used for these studies: (1) large injections into the caudate nucleus that spread to involve the thalamus, and (2) injections that involved only the caudate nucleus. The density of neurons in normal and ablated hemispheres for the large injections were as follows: II, 64 ± 7.1/64 ± 1.6; III, 59 ± 3.2/62 ± 1.0; Va, 61 ± 5.3/51 ± 2.8; Vb, 59 ± 6.4/42 ± 6.1* (i.e., −29%); VIa, 70 ± 6.2/16 ± 3.7* (i.e., −77%); and VIb, 30 ± 1.9/23 ± 2.1. Only alterations in layers Vb and VIa were significant (*). The

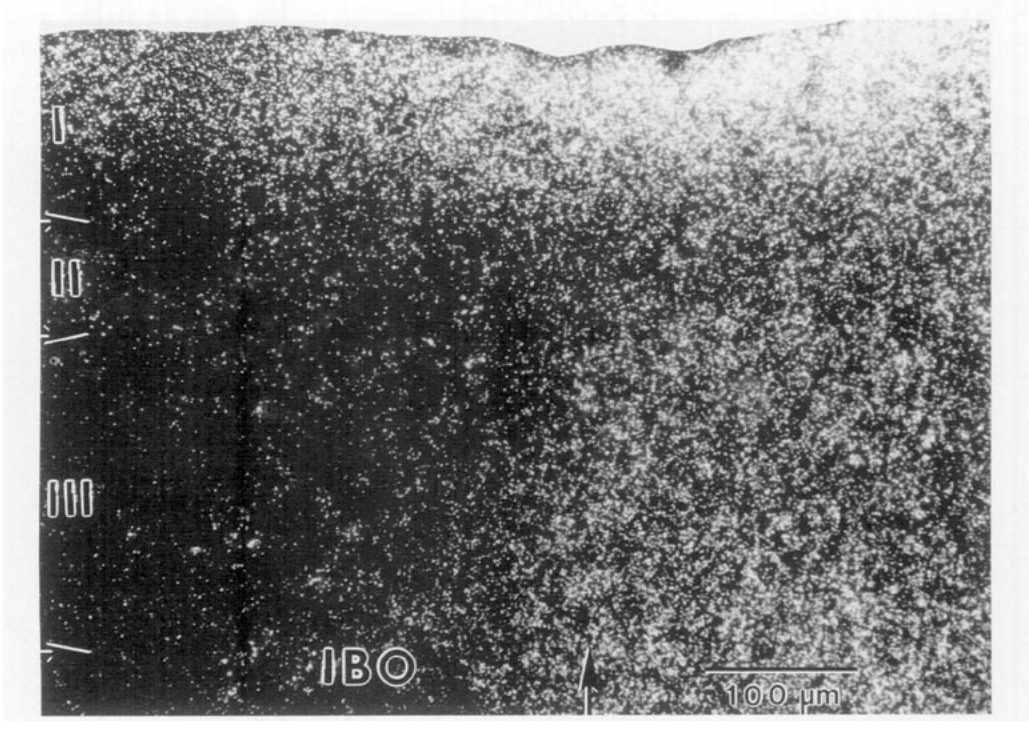


**FIG. 3.** Darkfield photomicrograph of the border (vertical arrow at bottom) of an ibotenic acid lesion (IBO) in area 24. The relatively high level of DAMGO binding in layers Ia and Ib is present in both the ibotenic acid-treated and the adjacent area 24.

injections limited to the caudate nucleus also produced significant changes only in layers Vb (62 ± 2.7/51 ± 1.8; −18%) and VIa (66 ± 6.7/39 ± 4.7; −41%). Since corticothalamic projection neurons have axons that traverse parts of the caudate nucleus, limited destruction of layer VI neurons in area 24 likely occurred as a consequence of retrograde transport of toxin in these axons.

OX7–saporin injections into the caudate and thalamic nuclei did not alter specific DPDPE in any layer of area 24 (Fig. 4). This was true in spite of a major reduction in the density of cortical projection neurons in layers Vb and VIa as shown in Fig. 5. The most pronounced neuron degeneration in this case was in layer VIa. Most of the neurons in layer VIb were preserved, as were some in layer Vb.

In contrast to DPDPE binding, there was a significant reduction in DAMGO binding in all layers of area 24 except layer II following OX7–saporin injections into the caudate and thalamic nuclei. An example of the reduced DAMGO binding in one of these cases is shown for the control and experimental hemispheres in the autoradiographs in Fig. 5.

Although injections of OX7–saporin in the caudate nucleus did not alter DPDPE binding, they had a limited influence on DAMGO binding. As shown in Fig. 6, there was a small reduction in specific DAMGO binding in all layers of area 24; however, only that in layer Vb was statistically significant.

## DISCUSSION

Area 24 undercut lesions reduced binding of DAMGO in layers I, III, and V without altering DPDPE binding, suggesting that there is a significant number of presynaptic mu but not delta opioid receptors in ACC. Excitotoxin lesions that destroy ACC neurons almost completely abolish binding of DPDPE and greatly reduce DAMGO binding. These findings suggest that all delta receptors are expressed by cortical neurons and that about half of mu receptors are postsynaptic and about half are presynaptic. Injections of the immunotoxin OX7–saporin into the thalamic and caudate nuclei greatly reduced DAMGO binding in layers V and VI but not DPDPE binding, while caudate nucleus injections reduced binding by a small amount only in layer V, the layer that contains the most cingulostriatal projection neurons. Thus, mu opioid receptors are expressed by ACC afferent axons and by neurons that project primarily to the thalamus and to a lesser extent to the caudate nucleus, while delta opioid receptors are synthesized by other cortical neurons.

One explanation that could account for reduced binding to mu receptors following undercut deafferentation lesions is a secondary transsynaptic change. There are two considerations that argue against this view. First, DPDPE binding was not altered in the same layers of the same cases, suggesting a receptor-specific change rather than a generalized transneuronal effect. Second, ibotenic acid injections remove all neurons and leave intact afferent axons in each layer, including layer I (44). High DAMGO binding in layer I after ibotenic acid lesions suggests that a major proportion of this binding is associated with preserved axon terminals. Thus, deafferentation lesions destroy axon terminals that express mu receptors, and reduced binding following these lesions cannot be accounted for by transsynaptic changes in postsynaptic neurons.

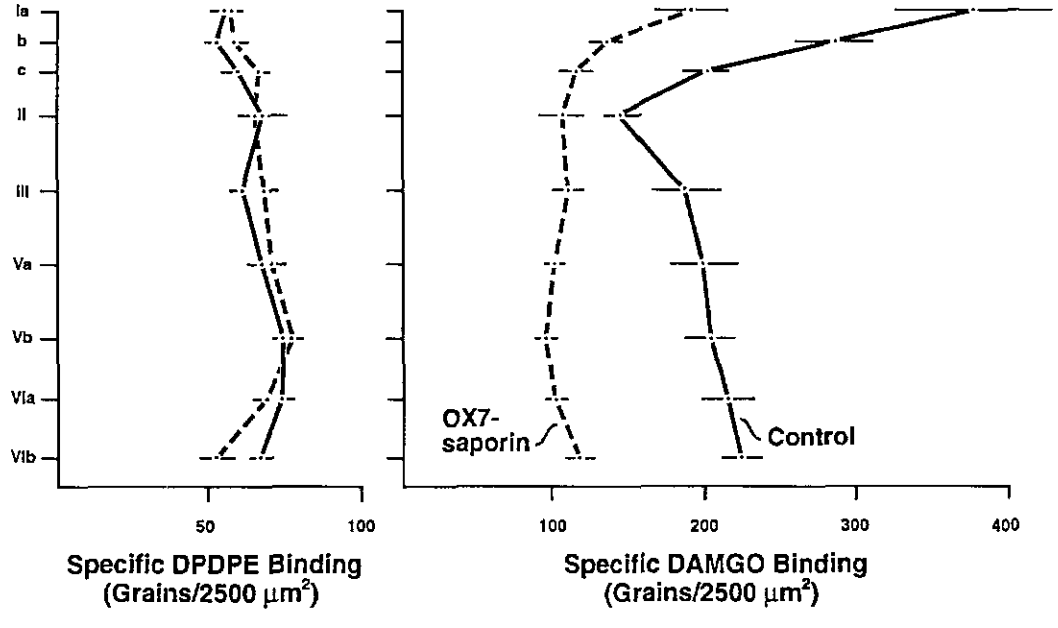


**FIG. 4.** Ligand binding in control and OX7–saporin-injected hemispheres from seven cases. The OX7–saporin was injected into the caudate and thalamic nuclei.

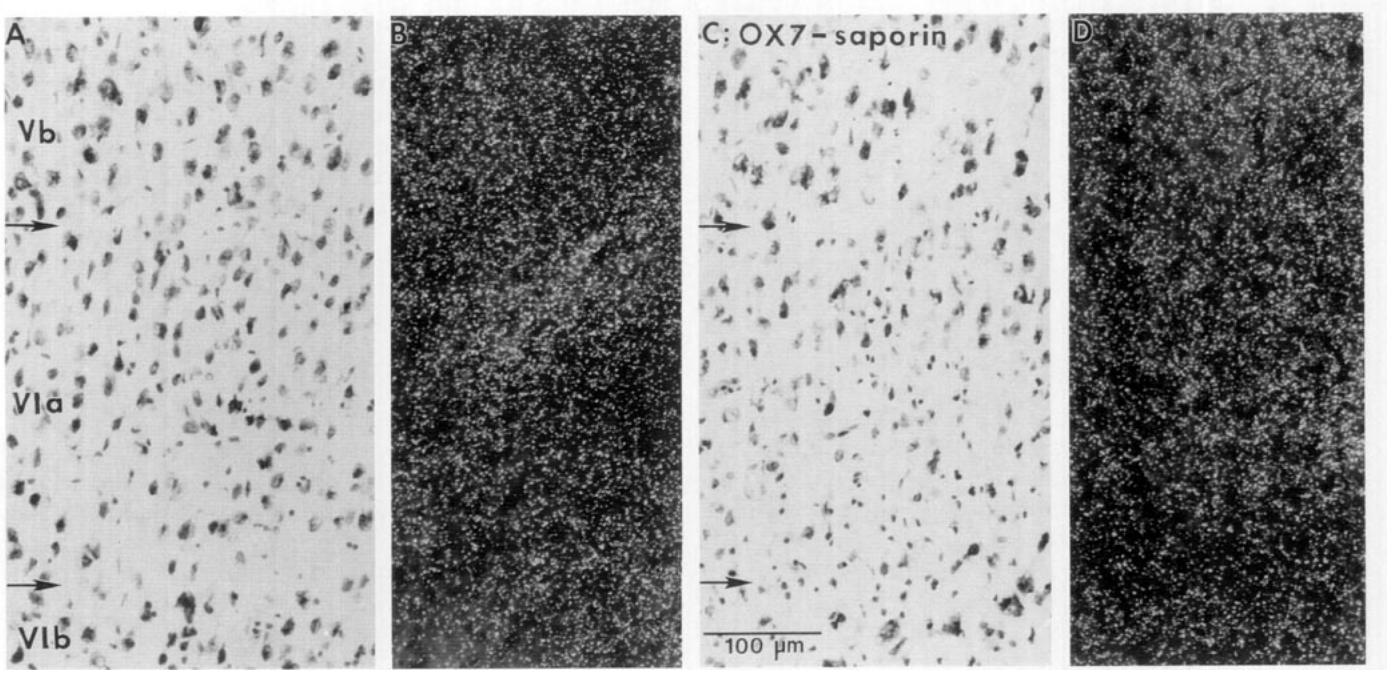


**FIG. 5.** Photomicrographs of Nissl-stained layers Vb–VI in area 24 of a control hemisphere (A) and one in which OX7–saporin was injected into the caudate and thalamic nuclei (C). The borders between layers Vb/VIa and VIa/VIb are identified with arrows in A and C. There are a few remaining neurons in layer VIb after the OX7–saporin injection which are likely corticocortical projection neurons (C). Examples of DAMGO binding in control (B) and OX7–saporin-injected (D) hemispheres is shown for the same layers in the darkfield photomicrographs for the same cases shown in A and B.

The present localization findings suggest an input/output model of mu opioid receptor regulation of ACC function. Figure 7 is a schematic of some of the key relationships in this model and raises issues relating to processing of nociceptive inputs and modulatory opioid circuits. One proviso should be restated here: although mu opioid receptors are widely distributed and not only

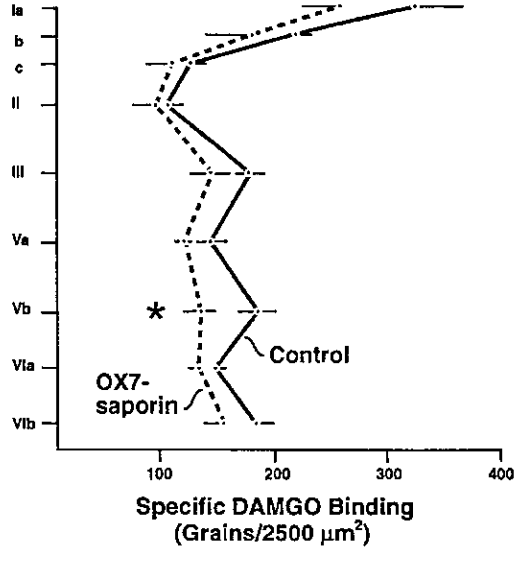


**FIG. 6.** Specific binding of DAMGO in control and OX7–saporin-injected hemispheres in four cases. Binding was reduced in all layers, but only in layer Vb (asterisk) was the reduction significant.

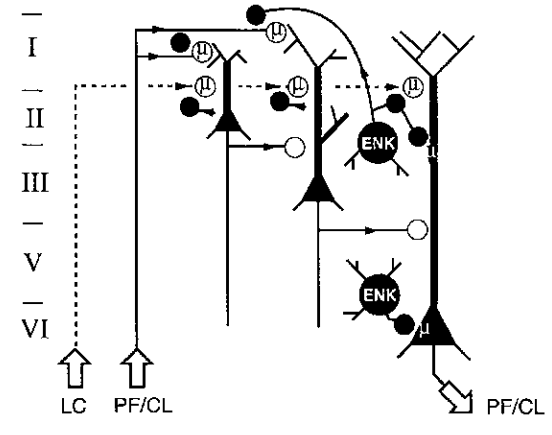


**FIG. 7.** Input/output model of mu opioid receptor regulation which emphasizes the thalamocortical loop through area 24. The circuits for processing nociceptive inputs include layer I afferents from the medial and intralaminar thalamic nuclei (PF/CL, parafascicular and centrolateral nuclei, respectively; open circles are for excitatory axon terminals) and activation of neurons in superficial layers II and III. Subsequent projections via axon collaterals to deep-layer neurons are postulated for regulation of responses to noxious stimuli mediated by brainstem structures including PF/CL. Opioidergic neurons (solid black somata and axon terminals, ENK) regulate this circuit at three critical points: (1) thalamic axon terminals in layer Ia/b, (2) electrical activity generated in layer I is gated by mu regulation of noradrenergic terminals in layers Ic and II (dashed lines; LC, locus coeruleus), and (3) postsynaptic receptors along the full somatodendritic region of corticothalamic projection neurons (i.e., in all layers).

engaged in nociception, in light of their high densities in ACC it is likely that one of their functions in this region is in modulating nociceptive activity.

*Nociceptive Circuit in ACC*

Most nociceptive neurons in area 24b are in layers II and III, and this activity can be blocked with lidocaine injections into the medial thalamus (38). Since neurons in the parafascicular (PF) and centrolateral (CL) thalamic nuclei are nociceptive (4, 11, 32) and they project to ACC (47), these are used in Fig. 7 as an example of nociceptive inputs to ACC. Other inputs that may be nociceptive and that receive spinothalamic inputs include the submedial, reuniens, paraventricular, and ventromedial thalamic nuclei (6, 7). Projections from the medial and intralaminar thalamic nuclei terminate in layer I (9, 17) and are so diagrammed in Fig. 7. Subsequent intrinsic processing of nociceptive inputs through axon collaterals of pyramidal neurons is not known, but a link to deep-layer projection neurons is necessary to engage descending corticobrainstem control (i.e., regulation of nociceptive thalamic nuclei and the periaqueductal gray). The relationship of ACC to the medial pain system has been recently assessed (49).

*Mu Regulation of Afferent Axon Terminals*

*In situ* hybridization studies of mRNA for the mu receptor have shown that brainstem nuclei with high levels of mu receptor synthesis are the mindline/intralaminar thalamic nuclei and the locus coeruleus (10, 26). The *in situ* studies show that the paraventricular, reuniens, parafascicular, and centrolateral thalamic nuclei contain neurons that synthesize mu receptors. Each of these nuclei receives spinothalamic inputs as discussed above, they project to ACC in the outer half of layer I (48), and they likely express high levels of mu receptors in layer Ia as diagrammed in Fig. 7. Although the present study did not employ selective thalamic lesions, there were significant alterations in DAMGO binding in layer Ia/b following undercut lesions, a layer that receives thalamic inputs (48), and posterior cingulate cortex mu opioid receptors are expressed by thalamic axon terminals in this layer (46). It should be noted parenthetically that the high level of layer Ia/b DAMGO binding is not unique to ACC, but occurs in lateral neocortical areas as well. Thus, it is likely that midline and intralaminar thalamic afferents to other cortical areas also express mu receptors.

Another mode of presynaptic regulation by mu receptors is via noradrenergic inputs which terminate mainly in the inner half of layer I in ACC (30). Noradrenaline release is reduced in the presence of mu agonists (31), and mu regulation of noradrenergic inputs to the deep part of layer I and layer II is shown in the input/output model in Fig. 7. Since noradrenaline inhibits neuronal discharges in the superficial layers of somatosensory cortex during peripheral stimulation (1), it is possible that the actions of opioids at the mu receptor are to gate the flow of thalamic activity from layer I to somata in deeper layers. One consequence of such a model is that the actions of mu agonists throughout the neocortex will differ depending on the extent of noradrenergic inputs to the deep part of layer I.

*ACC Opioidergic Neurons*

Opioidergic neurons are well documented in rat cerebral cortex, including ACC. There is a broad laminar distribution of preproenkephalin (16) and proenkephalin (28) neurons in cingulate cortex. Furthermore, immunoreactive neurons have a bipolar and multipolar morphology (28, 36), suggesting that endogenous opioid circuits originate in intrinsic neurons, not pyramidal neurons. Thus, the circuits shown in Fig. 7 incorporate multipolar local circuit neurons in the superficial and deep layers.

*ACC Neurons That Express Mu Receptors*

The *in situ* studies of mu receptor mRNA report that layer VI neurons express mu receptors (10, 26). The present findings showed that ablation of layer VI corticothalamic and layer V corticocaudate projection neurons with OX7–saporin reduced binding to mu receptors in all layers of ACC. Since selective caudate nucleus injections of OX7–saporin reduced binding only in layer Vb, it is concluded that the principal cause of reduced mu binding in the former larger lesions is loss of layer VI corticothalamic projection neurons. The broad laminar reductions in binding also suggest that corticothalamic projection neurons express mu receptors along the full length of their dendritic tree, and this feature is shown in Fig. 7. Finally, pyramidal neurons in layer VI project to the parafascicular nucleus (34) which is involved in responses to noxious stimuli (4, 32). The net changes in the output of the ACC circuit in the presence of opioid compounds cannot be predicted given the multiple pre- and postsynaptic locations of mu receptors.

Full-depth, area 24 ibotenic acid lesions in the present study almost abolished binding to mu receptors in layers II, III, and Va. Since the OX7–saporin injections removed almost all layer VIa neurons but not those in layers II and III, there is the possibility that some superficial pyramidal neurons also express mu opioid receptors. Although one *in situ* study did not mention mu receptor expression in ACC, it was suggested that cells in neocortical layers II–III expressed a low level of mu receptor that was not apparent in their autoradiographs (26). A study of the distribution of immunohistochemical labeling with an antibody to the mu opioid receptor showed receptors in the somata of layer II–III neurons in neocortex (2). Thus, although the input/output model presented in Fig. 7 emphasizes the thala-

mocortical loop, it is quite possible that neurons in superficial layers also express mu opioid receptors and these could include nociceptive neurons (38).

Most of what is known about the cellular actions of opioid receptors is derived from the hippocampal slice preparation. In this preparation, opioid compounds excite pyramidal neurons via a disinhibitory mechanism (24, 25). In view of the unique organizational features of the hippocampus and its lack of specific contributions to processing nociceptive inputs, it is not possible to generalize the functions of opioidergic circuits in the hippocampus throughout the cerebral cortex. In fact, the present findings and the *in situ* studies of mu receptor synthesis suggest localization of mu opioid receptors to major cortical inputs and outputs, not primarily to multipolar neurons.

### *Opioid Receptors and Chronic Pain Syndromes*

Intrinsic opioidergic circuits outlined above may be activated in conditions of chronic pain. Jones *et al.* (22) report that binding of diprenorphine in ACC of patients with rheumatoid arthritis that are in pain is significantly elevated over this binding in the same patients that have been treated and are no longer in pain. These authors suggest that elevated release of endogenous opioids leads to a reduction in *in vivo* binding of diprenorphine. If it is true that methionine enkephalin, for example, is elevated in chronic pain states, this compound is in a position to regulate ACC activity by inhibiting the release of glutamate from thalamocortical afferents, noradrenaline from afferents from the locus coeruleus, and by modulating the activity of cingulothalamic projection neurons.

The localizations of mu opioid receptors emphasize that, in order to understand opioid modulation of pain processing in the cerebral cortex, cortical areas that both are engaged in pain processing and have high levels of opioid receptors should be assessed. Studies limited to hippocampal preparations are inappropriate for this undertaking. Furthermore, the regulation of limbic cortical afferents by mu opioid receptors will require an assessment of the presynaptic actions of these compounds, particularly in relation to modulating the presynaptic release of glutamate from thalamic afferents.

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## REFERENCES

1. ARMSTRONG-JAMES, M., AND K. FOX. 1983. Effects of ionophoresed noradrenaline on the spontaneous activity of neurons in rat primary somatosensory cortex. *J. Physiol.* **335:** 427–447.
2. ARVIDSSON, U., M. RIEDL, S. CHAKRABARTI, J.-H. LEE, A. H. NAKANO, R. J. DADO, H. H. LOH, P.-Y. LAW, M. W. WESSENDORF, AND R. ELDE. 1995. Distribution and targeting of a μ-opioid receptor (MOR1) in brain and spinal cord. *J. Neurosci.* **15:** 3328–3341.
3. BALLANTINE, H. T., JR., B. S. LEVY, T. F. DAGI, AND I. B. GIRIUNAS. 1975. Cingulotomy for psychiatric illness: Report of 13 years' experience. Pages 333–353 in W. H. Sweet, S. Obrador, and J. G. Martin-Rodriguez, Eds., *Neurosurgical Treatment in Psychiatry, Pain, and Epilepsy.* University Park Press, Baltimore.
4. CASEY, K. L. 1968. Unit analysis of nociceptive mechanisms in the thalamus of the awake squirrel monkey. *J. Neurophysiol.* **29:** 727–750.
5. CASEY, K. L., S. MINOSHIMA, K. L. BERGER, R. A. KOEPPE, T. J. MORROW, AND K. A. FREY. 1994. Positron emission tomographic analysis of cerebral structures activated specifically by repetitive noxious heat stimuli. *J. Neurophysiol.* **71:** 802–807.
6. CRAIG, A. D., JR., AND H. BURTON. 1981. Spinal and medullary lamina I projection to nucleus submedius in medial thalamus: A possible pain center. *J. Neurophysiol.* **45:** 443–466.
7. CRAIG, A. D., JR., AND H. BURTON. 1985. The distribution and topographical organization in the thalamus of anterogradely-transported horseradish peroxidase after spinal injections in cat and racoon. *Exp. Brain Res.* **58:** 227–254.
8. CRINO, P. B., B. A. VOGT, L. VOLICER, AND R. G. WILEY. 1990. Cellular localization of serotonin 1A, 1B, and uptake sites in cingulate cortex of the rat. *J. Pharmacol. Exp. Ther.* **252:** 651–656.
9. CUNNINGHAM, E. T., AND S. LEVAY. 1986. Laminar and synaptic organization of the projection from the thalamic nucleus centralis to primary visual cortex in the cat. *J. Comp. Neurol.* **254:** 65–77.
10. DELFS, J. M., H. KONG, A. MESTEK, Y. CHEN, L. YU, T. REISINE, AND M.-F. CHESSELET. 1994. Expression of mu opioid receptor mRNA in rat brain: An in situ hybridization study at the single cell level. *J. Comp. Neurol.* **345:** 46–68.
11. DONG, W. K., H. RYU, AND I.H. WAGMAN. 1978. Nociceptive responses of neurons in medial thalamus and their relationship to spinothalamic pathways. *J. Neurophysiol.* **41:** 1592–1613.
12. FALLON, J. H., AND F. M. LESLIE. 1986. Distribution of dynorphin and enkephalin peptides in the rat brain. *J. Comp. Neurol.* **249:** 293–336.
13. FOLTZ, E. L., AND L. E. WHITE. 1968. The role of rostral cingulumotomy in "pain" relief. *Int. J. Neurol.* **6:** 353–373.
14. HARDY, S. G. P., AND G. R. LEICHNETZ. 1981. Cortical projections to the periaqueductal gray in the monkey: A retrograde and orthograde horseradish peroxidase study. *Neurosci. Lett.* **22:** 97–101.
15. HARDY, S. G. P., AND G. R. LEICHNETZ. 1981. Frontal cortical projections to the periaqueductal gray in the rat: A retrograde and orthograde horseradish peroxidase study. *Neurosci. Lett.* **23:** 13–17.
16. HARLAN, R. E., B. SHIVERS, G. J. ROMANO, R. D. HOWELLS, AND D. W. PFAFF. 1987. Localization of preproenkephalin mRNA in the rat brain and spinal cord by *in situ* hybridization. *J. Comp. Neurol.* **258:** 159–184.
17. HERKENHAM, M. 1979. The afferent and efferent connections of the ventromedial thalamic nucleus in the rat. *J. Comp. Neurol.* **183:** 487–518.
18. HILLER, J. M., J. PEARSON, AND E. J. SIMON. 1973. Distribution of stereospecific binding of the potent narcotic analgesic etorphine in the human brain: Predominance in the limbic system. *Res. Commun. Chem. Pathol. Pharmacol.* **6:** 1052–1062.
19. JONES, A. K. P., W. D. BROWN, K. J. FRISTON, L. Y. QI, AND R. S. J. FRACKOWIAK. 1991. Cortical and subcortical localization of re-

sponse to pain in man using positron emission tomography. *Proc. R. Soc. Lond. Biol.* **244:** 39–44.

20. JONES, A. K. P., K. J. FRISTON, L. Y. QI, M. HARRIS, V. J. CUNNINGHAM, T. JONES, C. FEINMAN, AND R. S. J. FRACKOWIAK. 1991. Sites of action of morphine in the brain. *Lancet.* **338:** 825.
21. JONES, A. K. P., L. Y. QI, T. FUJIWARA, S. K. LUTHA, J. ASHBURNER, P. BLOOMFIELD, V. J. CUNNINGHAM, M. ITOH, H. FUKUDA, AND T. JONES. 1991. *In vivo* distribution of opioid receptors in man in relation to the cortical projections of the medial and lateral pain systems measured with positron emission tomography. *Neurosci. Lett.* **126:** 25–28.
22. JONES, A. K. P., V. V. CUNNINGHAM, S. HA-KAWA, T. FUJIWARA, S. K. LUTHRA, S. SILVA, S. DERBYSHIRE, AND T. JONES. 1993. Changes in central opioid receptor binding in relation to inflammation and pain in patients with rheumatoid arthritis. *Br. J. Rheumatol.* **33:** 909–916.
23. KHACHATURIAN, H., M. E. LEWIS, V. HOLLT, AND S. J. WATSON. 1983. Telencephalic enkephalinergic systems in the rat. *J. Neurosci.* **4:** 844–855.
24. LUPICA, C. R., W. R. PROCTOR, AND T. V. DUNWIDDIE. 1992. Dissociation of μ and δ opioid receptor-mediated reductions in evoked and spontaneous synaptic inhibition in the rat hippocampus in vitro. *Brain Res.* **593:** 226–238.
25. MADISON, D. V., AND R. A. NICOLL. 1988. Enkephalin hyperpolarizes interneurons in the rat hippocampus. *J. Physiol. (Lond.)* **398:** 123–130.
26. MANSOUR, A., C. A. FOX, R. C. THOMPSON, H. AKIL, S. J. WATSON. 1994. μ-opioid receptor mRNA expression in the rat CNS: Comparison to μ-receptor binding. *Brain Res.* **643:** 245–265.
27. MANSOUR, A., H. KHACHATURIAN, M. E. LEWIS, H. AKIL, AND S. J. WATSON. 1987. Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *J. Neurosci.* **7:** 2445–2464.
28. MCGINTY, J. F., D. VAN DER KOOY, AND F. E. BLOOM. 1984. The distribution and morphology of opioid peptide immunoreactive neurons in the cerebral cortex of rats. *J. Neurosci.* **4:** 1104–1117.
29. MCLEAN, S., R. B. ROTHMAN, AND M. HERKENHAM. 1986. Autoradiographic localization of μ- and δ-opiate receptors in the forebrain of the rat. *Brain Res.* **378:** 49–60.
30. MORRISON, J. H., R. GRZANNA, M. E. MOLLIVER, AND J. T. COYLE. 1978. The distribution and orientation of noradrenergic fibers in neocortex of the rat: An immunofluorescence study. *J. Comp. Neurol.* **181:** 17–40.
31. MULDER, A. H., F. HOGENBOOM, G. WARDEH., AND A. N. M. SCHOFFELMEER. 1987. Morphine and enkephalins potently inhibit [$^3$H]noradrenaline release from rat brain cortex synaptosomes: Further evidence for a presynaptic localization of μ-opioid receptors. *J. Neurochem.* **48:** 1043–1047.
32. PESCHANSKI, M., G. GUILBAUD, AND M. GAUTRON. 1981. Posterior intralaminar region in rat: Neuronal responses to noxious and nonnoxious cutaneous stimuli. *Exp. Neurol.* **72:** 226–238.
33. ROYCE, G. J. 1982. Laminar origin of cortical neurons which project upon the caudate nucleus: A horseradish peroxidase investigation in the cat. *J. Comp. Neurol.* **205:** 8–29.
34. ROYCE, G. J. 1983. Cells of origin of corticothalamic projections upon the centromedian and parafascicular nuclei in the cat. *Brain Res.* **258:** 11–21.
35. SADZOT, B., J. C. PRICE, H. S. MAYBERG, K. H. DOUGLASS, R. F. DANNALS, J. R. LEVER, H. T. RAVERT, A. A. WILSON, H. N. WAGNER, M. A. FELDMAN, AND J. J. FROST. 1991. Quantification of human opiate receptor concentration and affinity using high and low specific activity [$^{11}$C]diprenorphine and positron emission tomography. *J. Cereb. Blood Flow Metab.* **11:** 204–219.
36. SAR, M., W. E. STUMPF, R. J. MILLER, K. J. CHANG, AND P. CUATRECASAS. 1978. Immunohistochemical localization of enkephalin in rat brain and spinal cord. *J. Comp. Neurol.* **182:** 17–38.
37. SCHWARCZ, R., T. HOKFELT, K. FUXE, G. JONSSON, M. GOLDSTEIN, AND L. TERENIUS. 1979. Ibotenic acid-induced neuronal degeneration: A morphological and neurochemical study. *Exp. Brain Res.* **37:** 199–216.
38. SIKES, R. W., AND B. A. VOGT. 1992. Nociceptive neurons in area 24b of rabbit anterior cingulate cortex. *J. Neurophysiol.* **68:** 1720–1732.
39. TALBOT, J. D., S. MARRETT, A. C. EVANS, E. MEYER, M. C. BUSHNELL, AND G. H. DUNCAN. 1991. Multiple representations of pain in human cerebral cortex. *Science* **251:** 1355–1358.
40. TODA, K. 1992. Anterior cingulate-induced inhibition of activities of descending periaqueductal gray matter neurons in rats. *Pain Res.* **7:** 71–79.
41. VACCARINO, A. L., AND R. MELZACK. 1989. Analgesia produced by injection of lidocaine into the anterior cingulum bundle of the rat. *Pain* **39:** 213–219.
42. VAN GROEN, T., B. A. VOGT, AND J. M. WYSS. 1993. Interconnections between the thalamus and retrosplenial cortex in the rodent brain. Pages 123–150 in B. A. Vogt and M. Gabriel, Eds., *Neurobiology of Cingulate Cortex and Limbic Thalamus.* Birkhauser Boston, Cambridge, MA.
43. VOGT, B. A. 1984. Afferent specific localization of muscarinic acetylcholine receptors in cingulate cortex. *J. Neurosci.* **4:** 2191–2199.
44. VOGT, B. A. 1993. Structural organization of cingulate cortex: Areas, neurons, and somatodendritic transmitter receptors. Pages 19–70 in B. A. Vogt and M. Gabriel, Eds., *Neurobiology of Cingulate Cortex and Limbic Thalamus.* Birkhauser Boston, Cambridge, MA.
45. VOGT, B. A., AND D. L. BURNS. 1988. Experimental localization of muscarinic receptor subtypes to cingulate cortical afferents and neurons. *J. Neurosci.* **8:** 643–652.
46. VOGT, B. A., P. B. CRINO, AND E. L. JENSEN. 1992. Multiple heteroreceptors on limbic thalamic axons: $M_2$ acetylcholine, serotonin$_{1B}$, $\beta_2$-adrenoceptors, μ-opioid, and neurotensin. *Synapse* **10:** 44–53.
47. VOGT, B. A., D. L. ROSENE, AND D. N. PANDYA. 1979. Thalamic and cortical afferents differentiate anterior from posterior cingulate cortex in the monkey. *Science* **204:** 205–207.
48. VOGT, B. A., D. L. ROSENE, AND A. PETERS. 1981. Synaptic termination of thalamic and callosal afferents in cingulate cortex of the rat. *J. Comp. Neurol.* **201:** 265–283.
49. VOGT, B. A., R. W. SIKES, AND L. J. VOGT. 1993. Anterior cingulate cortex and the medial pain system. Pages 313–344 in B. A. Vogt and M. Gabriel, Eds., *Neurobiology of Cingulate Cortex and Limbic Thalamus.* Birkhauser Boston, Cambridge, MA.
50. VOGT, B. A., H. WATANABE, S. GROOTOONK, AND A. K. P. JONES. 1995. Topography of diprenorphine binding in human cingulate gyrus and adjacent cortex derived from coregistered PET and MR images. *Hum. Brain Mapping* **3:** 1–12.
51. VOGT, L. J., B. A. VOGT, AND R. W. SIKES. 1992. Limbic thalamus in rabbit: Architecture, projections to cingulate cortex and distribution of muscarinic acetylcholine, $GABA_A$, and opioid receptors. *J. Comp. Neurol.* **319:** 205–217.
52. WILEY, R. G. 1992. Neural lesioning with ribosome-inactivating proteins: Suicide transport and immunolesioning. *Trends Neurosci.* **15:** 285–290.
53. WILEY, R. G., F. STIRPE, P. E. THORPE, AND T. N. OELTMANN. 1989. Neurotoxic effects of a monoclonal anti-Thy 1.1 antibody (OX7) coupled to the ribosome inactivating protein, saporin, as studied by suicide transport experiments in the rat. *Brain Res.* **505:** 44–54.