SPECIFIC BINDING OF NALOXONE TO OVINE BRAIN TISSUE: COMPARISON OF BRAIN REGIONS AND ENDOCRINE STATES

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ABSTRACT

Binding of \([^3H]\)naloxone ([^3H]NAL) to brain membranes was quantified by Scatchard analysis using two methods of separating bound from free [^3H]NAL. In the centrifugation method, membranes that were soluble at 1,000 \(\times\) g, but sedimented at 20,000 \(\times\) g, were incubated with [^3H]NAL. For filtration, all membranes that sedimented at 20,000 \(\times\) g were incubated and filtered through glass filter fibers. Nonspecific binding was estimated using >500-fold excess of unlabeled naloxone (10^{-6} M). Specific binding of [^3H]NAL was used to generate linear multiple-point Scatchard plots, which indicated a single class of high-affinity sites. In Exp. 1, 10 ovariectomized (OVX) ewes were injected with estradiol-17\(\beta\) alone or in combination with progesterone. Compared with OVX controls, these hormonal treatments did not affect binding of [^3H]NAL (centrifugation method) to combined hypothalamus (HYP) + preoptic (POA) tissues. In cyclic ewes (Exp. 2, filtration method), affinity constants (2.4 \(\pm\) 0.2 \(\times\) 10^{8} M^{-1}) did not differ among HYP, POA and basal forebrain (BF) tissues, but BF had more sites (39 \(\pm\) 3 fmol/mg) than either HYP (14 \(\pm\) 1) or POA (17 \(\pm\) 1). Binding affinity and concentration of sites within each brain area (HYP, POA, BF) did not differ between d 8 and d 16 (preovulatory but after luteolysis) in normally cycling ewes. Overall, neural tissue dissected from BF had a greater concentration of binding sites than HYP or POA. Exogenous and endogenous fluctuations in ovarian steroids did not affect binding of [^3H]NAL to these tissues.

(Key Words: Naloxone, Neurotropic Drugs, Receptors, Brain, Sheep, Steroids.)


Introduction

Endogenous opioid peptides (EOP) tonically inhibit release of LH from the pituitary gland. Administration of naloxone, an opioid receptor antagonist, increases LH release (i.e., disinhibits LH) in many experimental models and species (Brooks et al., 1986; Howlett and Rees, 1986; Malven, 1986; Kalra et al., 1987), providing essential support for the involvement of EOP in neuroendocrine regulation of LH release. Such naloxone-induced disinhibition of LH release requires the presence of 1) EOP agonists capable of inhibiting LH release and 2) functional opioid receptors somewhere in the pathways leading to LH release. Opioid receptors can be quantified in brain by various techniques (Terenius, 1973; Herkenham and Pert, 1982), but many of the measured receptors probably represent neural systems unrelated to regulation of LH release. By combining EOP receptor quantification with endocrine manipulations that modulate LH release, it may be possible to associate changes in EOP receptors more closely with neural regulation of LH release.

One objective of this research was to estimate opioid receptors by quantifying specific binding of naloxone in homogenates of ovine brain tissue. Because fluctuations in ovarian hormones altered the disinhibition of LH release by naloxone in ewes (Malven et al., 1984; Brooks et al., 1986; Currie and Rawlings, 1987; Trout and Malven, 1987), the
second objective was to relate changes in naloxone binding affinity or tissue concentration of binding sites to endocrine status.

**Materials and Methods**

**Experiment 1.** Brain tissue was obtained for dissection and binding analysis from 16 ovariectomized (OVX) ewes formerly designated Exp. 3 by Trout and Malven (1987). The following groups were represented: 1) control, no injections (n = 6); 2) daily s.c. injections of 50 µg estradiol-17β in oil for 5 d (n = 4); or 3) daily s.c. injections of estradiol-17β (50 µg) and progesterone (20 mg) in oil for 5 d (n = 6). After 5 d of treatment, ewes were injected i.v. with naloxone (1 mg/kg BW), followed 2 h later with LHRH (1 µg/ewe). Approximately 2 h after LHRH injection, ewes were rendered unconscious by an i.v. injection of sodium pentobarbital and were immediately exsanguinated. Each brain was removed, wrapped in foil and placed on ice. Within 1 h after removal, each brain was prepared for measurement of naloxone binding sites (Trout and Malven, 1988).

From each brain, a tissue fragment was dissected to include the preoptic area, the anterior hypothalamic area and the medial basal hypothalamus. This fragment was limited rostrally by the anterior commissure, caudally by the mammillary bodies, dorsally by the thalamus and laterally by the hypothalamic sulci. Finally, the optic chiasm was removed. The resulting tissue fragment, designated as hypothalamus (HYP) + preoptic area (POA), was weighed and homogenized in 50 mM Tris-HCl (pH 7.4) using a Brinkmann Polytron (speed 3.5 for 15 s). This homogenate was centrifuged at 1,000 x g for 20 min. After discarding the pellet, the supernatant fluid was centrifuged at 20,000 x g for 20 min. The resulting pellet was resuspended in an appropriate volume of Tris-HCl to yield a final tissue concentration of 20 to 40 mg of tissue wet weight per 1 ml aliquot. The naloxone binding assay was performed in polypropylene tubes in a total volume of .25 ml. Aliquots (.1 ml) of homogenate were incubated (2 h, room temperature) with one of the eight known concentrations of [3H]naloxone ([3H]NAL) ranging from 8,000 to 110,000 cpm (8 to 125 x 10^-10 M naloxone). Nonspecific binding was determined as the amount of [3H]NAL that bound in the presence of at least a 500-fold molar excess of unlabeled naloxone (10^-6 M). Nonspecific binding was subtracted from total binding to yield specific binding of [3H]NAL. Separation of [3H]NAL bound to plasma membranes from free [3H]NAL was achieved by centrifugation at 20,000 x g for 20 min followed by two washings each with 4 ml of Tris-HCl. The pellet was transferred to a scintillation counting vial with three 3.3-ml rinses of liquid scintillation cocktail. Specifically bound counts of [3H]NAL were subjected to multiple-point Scatchard analyses to estimate binding affinity and concentration of naloxone binding sites per milligram of original tissue.

**Experiment 2.** Because the results of Exp. 1 did not reveal any differences due to hormone treatment, another study was designed with the following improvements: 1) filtration replaced centrifugation for separation of bound and free [3H]NAL in order to allow smaller fragments of tissue to be assayed, 2) POA and basal forebrain (BF) tissues shown to contain opioid receptors that varied with postpartum reproductive states in cattle (Trout and Malven, 1988) and to be brain sites at which local infusions of naloxone disinhibited LH release in ewes (Malven, 1988) were dissected separately and 3) natural endocrine states in cyclic ewes were used to create naloxone-reversible states of LH suppression (Malven et al., 1984; Currie and Rawlings, 1987).

Eight mature ewes exposed to natural photoperiods were observed twice daily for estrous behavior during September and October. Four ewes each were killed on d 8 or d 16 (d 0 = estrus) approximately 3 h after being injected i.v. with naloxone (1 mg/kg BW). Serum concentrations of LH were quantified (Trout and Malven, 1987) at 10-min intervals 0 to 20 min before naloxone and 10 to 30 min after naloxone. Serum progesterone and LH were also quantified once daily for several days before death on d 16. All ewes killed on d 16 and included in this report were shown by hormonal analyses to have been after luteolysis and prior to the preovulatory surge of LH. Each ewe was rendered unconscious by a captive-bolt pistol applied to the occipital region and was immediately exsanguinated. Brain tissue was removed and immediately frozen at -80°C.

At the time of the naloxone binding assay, brains were thawed at room temperature and kept moist with Tris-HCl buffer. Each brain
TABLE 1. EFFECT OF EXOGENOUS OVARIAN STEROIDS ON SPECIFIC BINDING OF [3H]NALOXONE TO HOMOGENATES OF HYPOTHALAMUS + PREOPTIC AREA OF OVARIECTOMIZED (OVX) EWES (EXP. 1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of ewes</th>
<th>Affinity $K_a \times 10^8 M^{-1}$</th>
<th>Binding sites, fmol/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>6</td>
<td>$5.3 \pm 1.4^a$</td>
<td>$1.52 \pm 0.14$</td>
</tr>
<tr>
<td>OVX + estradiol-17β</td>
<td>4</td>
<td>$11.1 \pm 2.5$</td>
<td>$1.31 \pm 0.32$</td>
</tr>
<tr>
<td>OVX + estradiol-17β + progesterone</td>
<td>6</td>
<td>$8.3 \pm 2.1$</td>
<td>$1.45 \pm 0.32$</td>
</tr>
</tbody>
</table>

$^a$Mean ± SE.

was dissected to yield the following separate fragments: HYP, POA and BF. The HYP extended from the rostral edge of the mammillary bodies to the caudal edge of the optic chiasm. The POA tissue fragment extended from the caudal edge of the optic chiasm to 1 to 2 mm rostral to the rostral edge of the optic chiasm. The BF fragment was taken from the ventral surface of the brain and was bordered caudally by the POA fragment. It extended approximately 8 mm rostrally from the POA fragment to the midline separation of the hemispheres. Laterally, it was bounded by the basal ganglia and was assumed to include ventral parts of the septum, diagonal band of Broca, nucleus accumbens, and perhaps rostral areas of POA (Richard, 1967).

Each tissue was homogenized, centrifuged at 20,000 x g for 20 min, and diluted to 5 to 10 mg tissue wet weight per .1 ml Tris-HCl. Tissue was handled as described for Exp. 1 with the following two exceptions. First, the initial centrifugation of the homogenate at low speed (1,000 x g) was eliminated. Second, separation of bound [3H]NAL from the free [3H]NAL was achieved by low vacuum filtration through glass filter fibers* (effective retention = 1 micron). Filters were rinsed twice with 4 ml of Tris-HCl. The resulting filter residue, which contained the bound fraction, was added to 3.3 ml liquid scintillation cocktail and counted for radioactivity. Multiple-point Scatchard analyses were applied to these data.

In Exp. 1, mean concentrations of [3H]NAL binding sites, as well as mean binding affinities of these sites, were compared among treatment groups. In Exp. 2, mean concentrations and binding affinities of the binding sites were compared between different days of the estrous cycle, then pooled for comparisons among brain areas. All statistical analyses were performed by a General Linear Models procedure (SAS, 1985). Only when significant differences ($P < .05$) were detected by analysis of variance were Student-Newman-Keuls tests used for separation of means.

Results

For each homogenate, a multiple-point Scatchard plot was generated, as illustrated in Figure 1. Slope of the linear regression line fitted by least squares among data points was used to estimate the affinity constant ($K_a$) for each homogenate and its inverse, the dissociation constant ($K_d$). Extension of the linear line to intersect with the abscissa was used to estimate the total amount (fmol) of bound naloxone theoretically required for saturation

Figure 1. Example of Scatchard plot used to estimate the dissociation constant ($K_d$) and the theoretical saturation of receptors for one brain tissue homogenate. The ordinate represents the ratio of bound [3H]naloxone ([3H]NAL) to free [3H]NAL, and the abscissa is the amount (cpm) of bound [3H]NAL. The solid line denotes the linear regression fitted by least squares to the eight data points. The dashed line denotes the extension of the regression line to intersect with the abscissa.

*Whatman GF/B, Fisher Scientific Co., Itasca, IL.
of all specific binding sites in that homogenate. Administration of ovarian steroids to OVX ewes in Exp. 1 had no effect on either the binding affinity or concentration of naloxone binding sites in tissues representing combined HYP and POA tissue (Table 1). Across all treatment groups, binding affinity averaged 7.9 ± 2.3 (× 10⁶ M⁻¹), and concentration of specific naloxone binding sites averaged 1.43 ± .27 fmol per milligram of tissue wet weight.

Quantification of naloxone binding in cycling ewes of Exp. 2 is given in Table 2 and Figure 2. Binding affinity did not differ (P > .05) among brain areas or days of the estrous cycle (Table 2). Concentration of specific binding did not differ between d 8 and d 16 for any brain area (Figure 2). However, when pooled across days, homogenates of BF contained greater (P < .01) concentrations of sites (39 ± 3.1 fmol/mg) than did either HYP (13.6 ± .8 fmol/mg) or POA (16.7 ± .9 fmol/mg).

Serum LH during the 20 min immediately before i.v. injection (pre-naloxone) was, as expected, lower (P < .02) on d 8 than d 16 (.27 ± .01 vs 1.58 ± .48 ng/ml). When post-naloxone (10 to 30 min after injection) LH was compared with pre-naloxone, ewes on d 8 exhibited an increase (P < .05) from .27 ± .01 to 7.15 ± 2.73 ng/ml. However, serum LH in ewes on d 16 only tended to increase (P < .10) from pre- to post-naloxone (1.58 ± .48 vs 3.12 ± .69 ng/ml). Post-naloxone concentrations of LH did not differ between d 8 and d 16 due in part to very large variation.

**Discussion**

In Exp. 1, only one fragment of brain tissue (i.e., HYP+POA) per ewe could be analyzed for naloxone binding using multiple-point Scatchard procedures. This limitation was imposed by the quantity of tissue required in each assay tube (20 to 40 mg) for the centrifugation method for separation of bound [³H]NAL from free [³H]NAL. For Exp. 2, vacuum filtration was used as the separation procedure, and it allowed smaller quantities of tissue (5 to 10 mg per tube) to be studied. Although the two procedures were not compared statistically because animals and brains were prepared and handled differently, the filtration method appeared consistently to detect more binding sites per milligram of original tissue. It is likely that in discarding the pellet from the 1,000 × g centrifugation many naloxone binding sites were discarded.

Vacuum filtration allowed assay of BF tissue fragments, and this area of brain was of interest for several reasons. Immunocytochemical evaluation of ovine brain has demonstrated that LHRH cell bodies are present within this brain area (Lehman et al., 1986; Caldini et al., 1988). Likewise, autoradiographic studies revealed a relatively high concentration of opioid binding sites in the basal forebrain area of several nonruminant species (Atweh and Kuhar, 1977; Ronai, 1983). The present results, obtained with in vitro binding of naloxone to
tissue homogenates, confirm this observation for sheep. It would be of great interest to determine whether LHRH cell bodies actually possess opioid receptors, or if the receptors are located on some regulatory interneuron. The recent observation of β-endorphin synapses on LHRH neurons in male rats (Chen et al., 1988) is consistent with the first possibility.

Brain areas rostral to the POA, which were designated collectively as BF in this paper, contained more naloxone binding sites than other regions. Basal forebrain tissue dissected from the brains of rams, wethers, and various steroid-treated ewes consistently has yielded greater concentrations of naloxone binding sites than corresponding HYP and POA tissues (unpublished data). In rats, one of the prominent structures within the present BF fragment, the nucleus accumbens, also contains high levels of mu, delta, and kappa subtypes of the opioid receptor (Mansour et al., 1987; Tempel and Zukin, 1987). Furthermore, the BF and POA regions of ewes responded to locally infused naloxone with disinhibition of LH release (Malven, 1988).

Neither binding affinity nor concentration of binding sites was affected by estradiol-17β and progesterone in OVX ewes using the centrifugation method. However, these treatments did not create the intended differential states for naloxone-reversible inhibition of LH release. Combined administration of estradiol-17β and progesterone suppressed serum LH more than estradiol-17β alone (see Table 2 of Trout and Malven, 1987, which represents the same ewes), but they did not create a significant naloxone-reversible inhibition of LH. Intact ewes killed on either d 8 or d 16 of the estrous cycle did not differ in naloxone binding characteristics, despite the fact that d-8 ewes exhibited more naloxone-reversible inhibition of LH release than d-16 ewes, in agreement with Currie and Rawlings (1987). It must be acknowledged that the present dissection of brain areas was not histologically verified for each animal. Variation in accuracy of dissection may make hormonal effects on naloxone binding more difficult to detect.

In contrast to present results, opioid receptor sites have been observed to differ between certain reproductive states. Among suckled beef cows, anestrous cows had higher concentrations of naloxone binding sites in tissues of POA and BF than did cyclic cows (Trout and Malven, 1988). Limonta et al. (1987) observed a significant increase in concentration of hypothalamic mu opioid receptors (estimated by dihydromorphine binding) in cyclic female rats between 1000 and 1200 on the day of proestrus. Wilkinson et al. (1985) observed that estradiol implants increased naloxone binding in the anterior hypothalamus of OVX rats, whereas Vertes et al. (1986) observed that estradiol injections decreased naloxone binding capacity in the hypothalamus of OVX rats.

With the present [3H]NAL as a binding ligand, it was not possible to determine experimentally which subtypes of the opioid receptor were being quantified. However, mu, delta and perhaps epsilon subtypes were all possible contributors to the present binding of [3H]NAL. It might be argued that the large amount of [3H]NAL binding to neural systems unrelated to regulation of LHRH/LH masks any hormone-induced changes in opioid modulation of LHRH/LH. Nevertheless, naloxone-reversible inhibition of LH release in ewes of differing hormonal status did not appear to be related to changes in the present measurements of naloxone binding. However, Exp. 1 failed to produce the intended differences in naloxone reversibility of LH release. Therefore, the relationship could be properly tested only in Exp. 2, and the lack of differences in naloxone binding suggested that changes in opioid receptors do not mediate the changes in naloxone-reversible progesterone-induced inhibition of LH release. Perhaps changes in secretion of EOP agonists are involved, as suggested for β-endorphin secretion in monkeys exposed to different hormonal environments (Ferin et al., 1984).

**Literature Cited**


NALOXONE BINDING SITES IN OVINE BRAIN


