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Estrogen Regulation of $GABA_A$ Receptor Subunit mRNA Expression in Preoptic Area and Bed Nucleus of the Stria Terminalis of Female Rat Brain

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This study has examined whether circulating estrogens are involved in regulating γ-aminobutyric acid $(GABA)_A$ receptor mRNA expression in regions of the female rat brain known to contain estrogen receptors (ERs). *In situ* hybridization experiments using ^{35}S-labeled oligonucleotides specific for α_2, β_3, and γ_1 subunit mRNAs of the $GABA_A$ receptor demonstrated that all three mRNAs were abundant in only the medial preoptic nucleus (MPN), where they were expressed by the vast majority of cells, and specific regions of the bed nucleus of the stria terminalis including the principle encapsulated nucleus (PrN-BNST) and bed nucleus of the anterior commissure (BNAC). Estrogen treatment of ovariectomized rats for 7 d resulted in significant 30–60% increases in α_2 and γ_1, but not β_3, subunit mRNA expression in the MPN and PrN-BNST. Estrogen treatment for 24 hr resulted in levels of mRNA expression intermediate between those of controls and animals treated with estrogen for 7 d. No changes in subunit mRNA expression were detected for any subunit in the BNAC or cingulate cortex. Double-labeling immunocytochemistry experiments using antibodies directed against the α_2 subunit of the $GABA_A$ receptor and the ER, revealed that 67 ± 3% of α_2 subunit-immunoreactive cells in the MPN also contained ER immunoreactivity. Cells expressing α_2 subunits in the PrN-BNST were also found to possess ERs while those in the BNAC and cingulate cortex did not. These findings suggest the possibility that ER-containing cells in the MPN and PrN-BNST express an $\alpha_2\beta_3\gamma_1$ isoform of the $GABA_A$ receptor that has its α_2 and γ_1 subunits regulated by circulating estrogen concentrations. Together, our observations indicate that estrogen may regulate $GABA_A$ receptor mRNA expression at a transcriptional level and that this is only likely to occur within regions of the rat brain possessing ERs.

[Key words: bed nucleus of the stria terminalis, estrogen, estrogen receptors, GABA, $GABA_A$ receptor subunits,* in situ *hybridization, immunocytochemistry, preoptic area]

GABA acts on chloride ion-gated $GABA_A$ receptors to inhibit the electrical activity of neurons throughout the adult mammalian brain. Molecular cloning has identified at least five distinct classes of subunits, each with multiple subtypes (α_{1-6}, β_{1-4}, γ_{1-3}, δ, and ρ_{1-2}), which are believed to be arranged in a heteropentameric fashion to produce a variety of pharmacologically distinct isoforms of the $GABA_A$ receptor (Olsen and Tobin, 1990; Luddens and Wisden, 1991; Nayeem et al., 1994). Although the precise subunit composition of a single $GABA_A$ receptor has yet to be determined *in vivo*, investigators have begun to dissect the functions of different subunits within the GABA receptor; the presence of specific α and β subunits in recombinant hetero-oligomers have different effects on channel activity and ligand binding to the receptor and, in association with γ subunits, determine its pharmacological profile (Pritchett et al., 1989; Sigel et al., 1990; Ymer et al., 1990; McKernan et al., 1991; Amin and Weiss, 1993; Bureau and Olsen, 1993; Wafford et al., 1993).

Substantial evidence accumulated over the last decade indicates that steroid hormones are important regulators of $GABA_A$ receptor function. In particular, interest has focused on the direct actions of progesterone metabolites on $GABA_A$ receptors and their likely physiological role in modulating GABA actions at this receptor (Majewska, 1992; Paul and Purdy, 1992). In addition, however, estrogen has been implicated in the regulation of ligand binding to the $GABA_A$ receptor (Maggi and Perez, 1986; Lasaga et al., 1988; O'Connor et al., 1988; Schumacher et al., 1989a,b; Canonaco et al., 1993), although there is no evidence to suggest this steroid is a direct allosteric modulator of $GABA_A$ receptor. Further, as estrogen's influence on $GABA_A$ receptor ligand binding is restricted primarily to regions of the brain possessing estrogen receptors (ERs; O'Connor et al., 1988; Schumacher et al., 1989b; Canonaco et al., 1993), it is plausible that estrogen alters gene transcription to influence the $GABA_A$ receptor. To this end it is noteworthy that the preoptic area (POA) and bed nucleus of the stria terminalis (BNST) of the rat possess large populations of cells containing ERs (Cintra et al., 1986; Simerly et al., 1990) and are regions of the brain where estrogen alters the binding of ^{35}S-butylbicyclophosphorothioate (TBPS) but not ^3H-muscimol (O'Connor et al., 1988; Canonaco et al., 1993). Although the POA has been reported to express mRNAs for the $\alpha_{1,2,3,5}$, β_{1-3}, and $\gamma_{1,2}$ subunits of the $GABA_A$ receptor (Araki and Tohyama, 1992; Wisden et al., 1992; Petersen et al., 1993a), the autoradiograms of Wisden and colleagues (1992) show that the α_2, β_3, and γ_1 subunit mRNAs of the $GABA_A$ receptor are the only ones to be expressed in any strength within the medial preoptic nucleus (MPN) of the POA and the principle encapsulated nucleus of the BNST (PrN-BNST; Wisden et al., 1992); the two nuclei in which ER-containing cells are concentrated (Cintra et al., 1986; Simerly et al., 1990; Herbison and Theodosis, 1992). As such, these three $GABA_A$ re-

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ceptor subunits are good candidates for transcriptional regulation by the ER in the POA and BNST. Using *in situ* hybridization techniques, we have addressed this issue by examining the distribution of cells possessing mRNAs for α_2, β_3, and γ_1 subunits in the POA and BNST and determining the effects of estrogen on α_2, β_3, and γ_1 subunit mRNA expression in these areas. In addition, using antibodies that detect the ER and $GABA_A$ receptor α_2 subunit proteins, double-labeling immunocytochemistry techniques have been employed to assess whether α_2 subunits are, indeed, expressed by ER-immunoreactive cells in the POA and BNST.

Materials and Methods

Adult female Wistar rats from the Babraham colony were maintained in a light and temperature-controlled environment (lights on 0500–1900 hr, 22°C) with food and water freely available. All ovariectomies were undertaken on animals anesthetized with Avertin (2% tribromoethanol, 1.0 ml/100 gm body weight, i.p.) and experiments were conducted in accordance with Home Office requirements for Animal Scientific Procedures (Project 80/00704).

In situ *hybridization experiments.* Three groups of rats were ovariectomized and used 4 weeks later for *in situ* experiments. The first group of animals (OVX; $n = 5$) received subcutaneous implants of empty SILASTIC capsules (Dow Corning; length 14 mm, i.d. 0.062 in, o.d. 0.125 in; Dow Corning, USA) 1 week before being killed. The second group (OVX-E7d; $n = 5$) received SILASTIC implants filled with a 50 μg 17-β estradiol/ml ethyl oleate solution at the same time, while a third group (OVX-E1d; $n = 5$) received the same estradiol-filled capsule but 24 hr prior to decapitation. Estradiol capsules prepared and administered in this way return plasma estradiol concentrations to physiological levels observed at metestrous within 24 hr in female rats (Leipheimer et al., 1984). Between 1000 and 1200 hr, all rats were stunned by a blow to the back of the neck and decapitated, their brains removed, and frozen on dry ice. Fresh frozen sections (15 μm thick) were cut in the coronal plane through the POA and anterior hypothalamic area, thaw mounted onto gelatinized slides, and stored at −70°C.

Antisense oligonucleotides (42–45-mer) complementary to the coding regions for amino acids 340–354, 382–396, and 341–354 of the rat $GABA_A$ receptor α_2, β_3, and γ_1 subunits, respectively, were synthesized. These exact probe sequences have been used by others to assess α_2, β_3, and γ_1 subunit mRNA expression in the rat brain and the α_2 and β_3 specificity verified further using Northern blot analysis (Khrestchatisky et al., 1991; Ymer et al., 1989, 1990; Wisden et al., 1992). Each probe (100 ng) was 3′ end-labeled to a specific activity of approximately 10^9 cpm/μg by incubation with ^{35}S-dATP (1000–1500 Ci/mmol; New England Nuclear NEG 034H) and 50 U terminal deoxynucleotidyl transferase (Pharmacia, UK) in tailing buffer at 37°C for 30 min. Radiolabeled probes were purified by gel filtration on a Sephadex G-50 column. Brain sections from all three experimental groups were processed simultaneously for each oligonucleotide. Frozen sections were warmed to room temperature with a hair dryer and fixed in 4% paraformaldehyde/ 0.1 M phosphate buffer for 20 min. Sections were then rinsed in 0.1 M phosphate-buffered saline before being dehydrated through a series of graded ethanols, rehydrated in 95% ethanol, and finally left to air dry at room temperature. For hybridization, ^{35}S-labeled probes were diluted in hybridization buffer (50% deionized formamide, 4× SSC, 10% dextran sulphate, 1× Denhardt's solution, 250 μg/ml sheared salmon testis DNA, 3% β-mercaptoethanol) to a final concentration of approximately 2×10^3 cpm/μl, and 250 μl of diluted probe was applied to each slide containing four coronal brain sections. Following an overnight hybridization at 37°C, sections were rinsed in 1× SSC at room temperature (5 min), three times at 55°C (30 min each), and then for 1 hr at room temperature. After a brief rinse in dH_2O followed by 70% ethanol/300 mM ammonium acetate (30 sec) and then absolute alcohol (30 sec), sections were left to air dry before being apposed to Hyperfilm β-Max (Amersham) for 3 weeks. Slides were then dipped in Ilford K-5 nuclear track emulsion and exposed for 8–10 weeks in dark-tight boxes. After developing with Ilford Phenisol (1:5, 5 min at 20°C), slides were counterstained lightly with methylene blue and coverslipped. Signal specificity was assessed by use of competition experiments in which radiolabeled probes were hybridized to sections in the presence of a 25-fold excess of unlabeled probe.

Immunocytochemical studies. The monoclonal rat H222 antibody is directed against the hormone binding domain of the ER and, in rats, stained nuclei are only clearly detected in ovariectomized animals (Cintra et al., 1986; Herbison and Theodosis, 1992). Five rats were ovariectomized and perfused 10 d latter. All animals were deeply anesthetized with Avertin and perfused intracardially with heparinized saline (25 IU heparin/ml) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.6) for 10–15 min. Brains were then removed and a block containing the POA and BNST postfixed in the same 4% paraformaldehyde solution for 1–2 hr before being transferred into a 0.05 M Tris-buffered saline (TBS) containing 25% sucrose at 4°C. Coronal sections (25 μm) were taken through the POA and anterior hypothalamic area, washed several times in TBS, and then placed in 1% normal sheep serum/TBS for 1 hr at room temperature. For $GABA_A$ receptor α_2 subunit immunostaining, sections were incubated with an affinity purified polyclonal rabbit antisera (3 μg/ml) recognizing amino acids 416–424 of the α_2 subunit overnight at 4°C (Gift of W. Sieghart, Vienna). The next day sections were washed in TBS and incubated for 90 min in biotinylated goat anti-rabbit immunoglobulins (1:200, Vector UK) followed by streptavidin-biotin-peroxidase complex (1:200, Amersham UK) for another 90 min. Immunoreactivity was visualized using a nickel-enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB) procedure (Shu et al., 1988). Immunostaining for the ER, and double labeling was carried out on sections from ovariectomized rats as described previously (Herbison and Theodosis, 1992). In brief, the monoclonal rat H222 antibody directed against the human ER (Gift of Abbott Labs., Chicago, IL) was incubated with sections for 40 hr at a 1.3 μg/ ml concentration. Immunoreactivity was visualized using biotinylated anti-rat immunoglobulins (1:200, Amersham, UK) followed by the nickel-DAB procedure outlined above. Sequential double staining of sections was carried out by selecting ER-stained sections, washing them in TBS followed by a 40% methanol, 1% H_2O_2-TBS solution for 15 min, and incubation in the α_2 subunit antiserum as described. Immunoreactivity was visualized using peroxidase-labeled anti-rabbit immunoglobulins (3 hr, 1:200; Vector, UK) and DAB without nickel as the chromogen. All immunolabels were diluted in TBS containing 0.25% BSA with 0.3% Triton-X. The production and specificity of each antiserum has been reported previously (Greene et al., 1980; Cintra et al.,1986; Buchstaller et al., 1991; Zezula et al., 1991; Zimprich et al., 1991). For the α_2 subunit antibody, a solid phase absorption control was performed by placing POA sections with α_2 antiserum previously incubated with 140 μg of the α_2 nonapeptide (gift of W. Sieghart) absorbed on CNBr-activated Sepharose-4B beads (0.25 gm overnight at 4°C, Pharmacia, UK). Other controls consisted of the omission of primary antibodies and incubation of sections in inappropriate secondary antibodies. In all control experiments, no specific staining was evident.

Data analysis. For *in situ* hybridization experiments, relative numbers of silver grains overlying individual cells in the medial division of the medial preoptic nucleus (MPN), PrN-BNST, and layers 2 and 3 of the cingulate cortex were determined using a Joyce-Loebl Magiscan analyzer coupled to a Leica Orthoplan microscope. In each of these regions, 20–25 labeled cells were analyzed from both sides of the brain of each animal. In relatively cell-dense regions such as the MPN and PrN-BNST, care was taken to analyze only those cells for whom the silver grains were clearly attributable to a single cell. Initially, the numbers of silver grains overlying cells in the excess unlabeled probe control sections were determined and in experimental sections, only those cells expressing numbers of silver grains greater than 5 times that of controls were used for analysis. For each rat and each region an average silver grain count/cell was determined and these values combined to give experimental group means. Silver grain analysis was also undertaken in the bed nucleus of the anterior commissure (BNAC) within the BNST, although the very high density of cells in this region precluded any individual cell analysis. Instead, silver grain density was determined using the Joyce-Loebl Magiscan by counting the numbers of silver grains overlying defined areas of the BNAC in two to three sections from each rat and represented as silver grains/μm². In all cases, statistical analysis was determined using ANOVA followed by the nonparametric Mann–Whitney U test.

A semiquantitative analysis of double-labeled immunoreactive cells was carried out by counting the numbers of ER-, α_2 subunit- and double-labeled cells in the MPN of ovariectomized rats using a Leica DM RB microscope at 40× objective magnification. For each rat, two to four sections through the MPN at the level depicted in Figure 5, were analyzed by counting all immunoreactive cells within a 1 mm by 1 mm reference frame placed over the MPN.

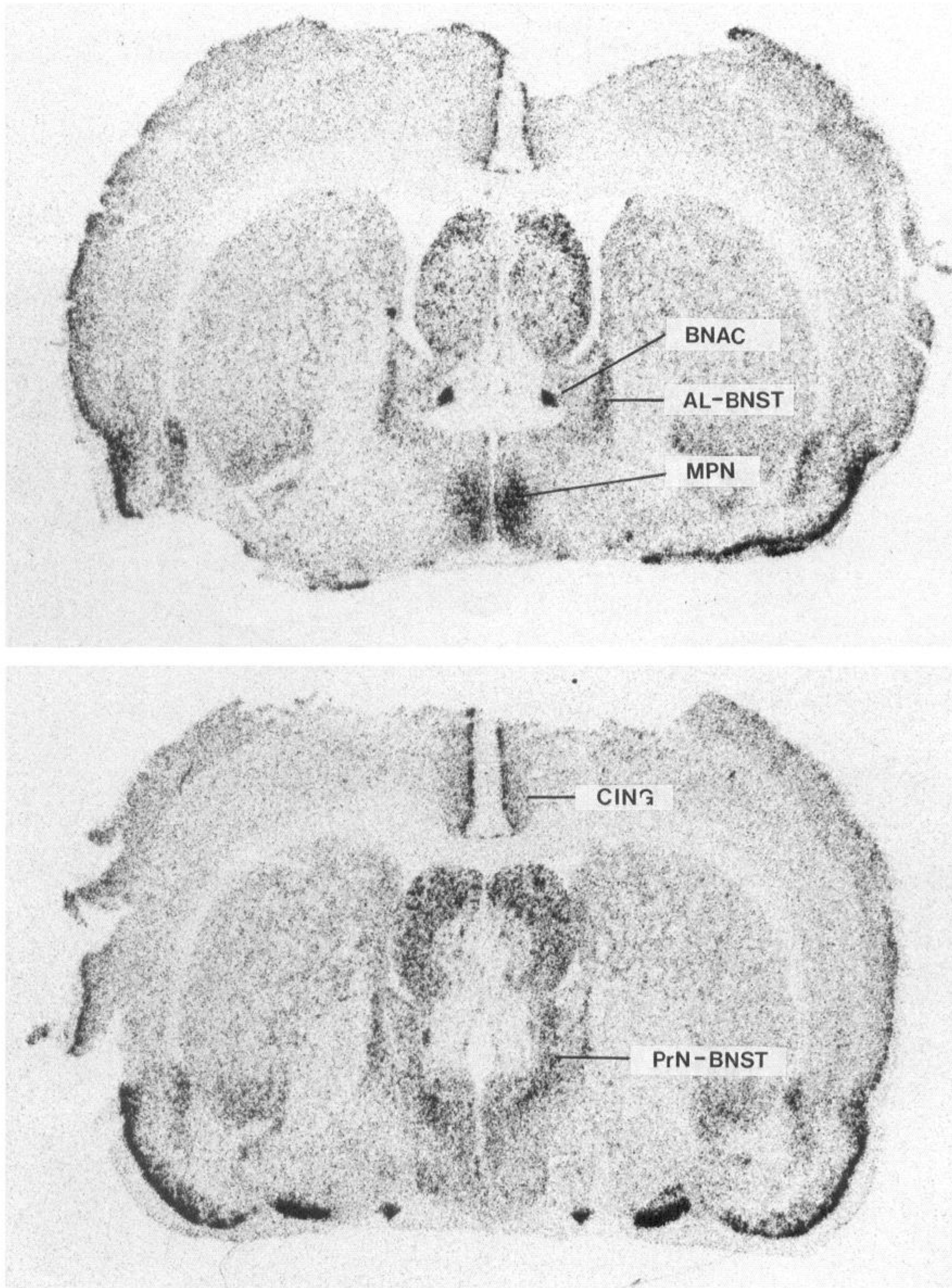

Figure 1. Distribution of $GABA_A$ receptor α_2 subunit mRNA transcripts in coronal sections of the female rat brain taken through the medial preoptic nucleus (*top*) and anterior hypothalamic area (*bottom*). *AL-BNST,* anterolateral division of the bed nucleus of the stria terminalis; *BNAC,* bed nucleus of the anterior commissure; *CING,* cingulate cortex; *MPN,* medial preoptic nucleus; *PrN-BNST,* principle encapsulated nucleus of the bed nucleus of the stria terminalis.

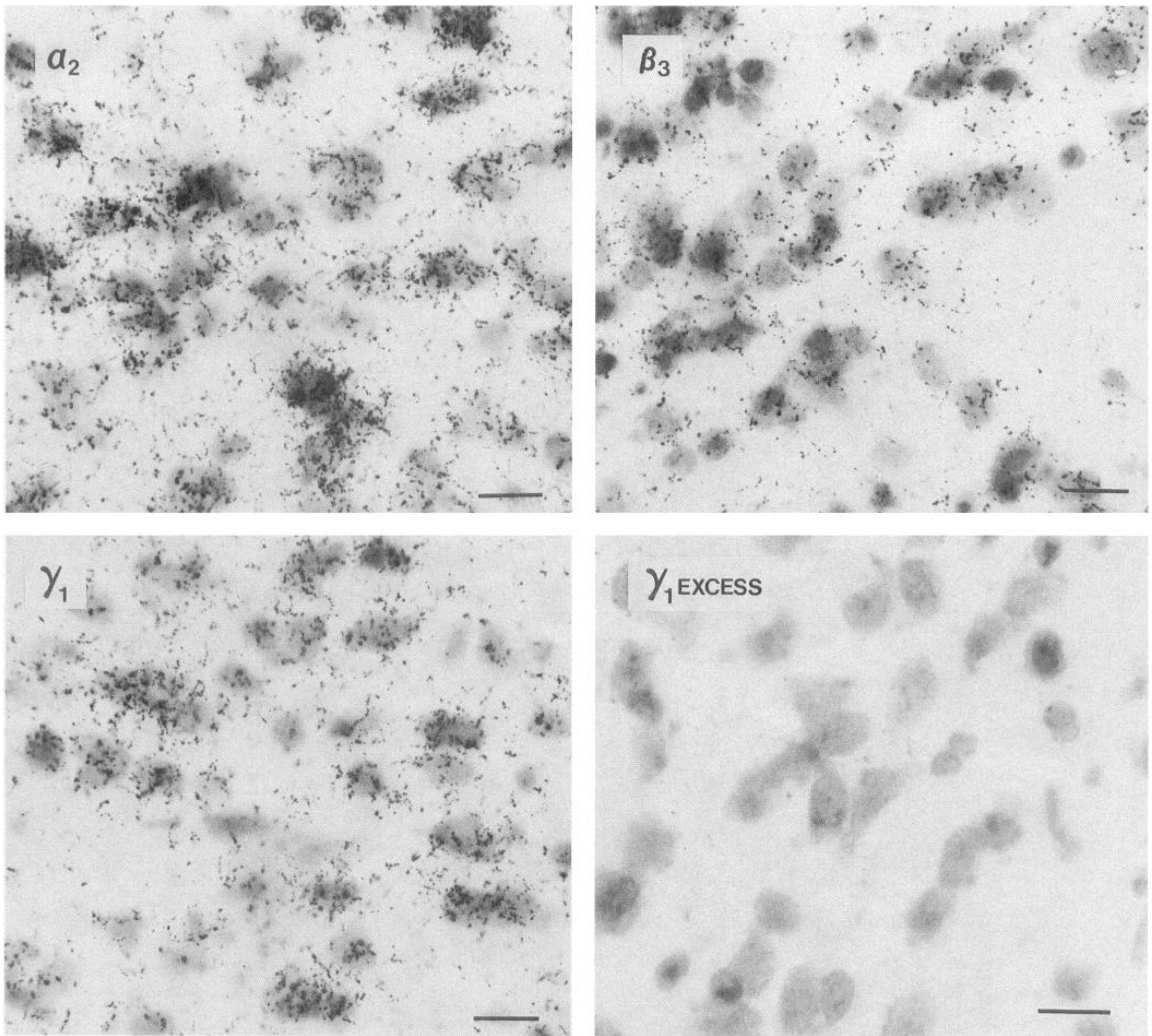

Figure 2. High-power views of cells in the medial preoptic nucleus expressing α_2, β_3, and $\gamma 1$ subunit mRNAs. γ_1 *EXCESS* shows hybridization signal following excess unlabeled probe control experiments (see Materials and Methods). Note that most cells express the β_3 subunit while almost all cells contain α_2 and γ_1 subunit mRNA transcripts. Scale bars, 15 μm.

Results

In situ *hybridization experiments*

The distribution of hybridized probe was first assessed by film autoradiography and then verified by examining silver grain distribution and the intensity of labeling recorded (heavy +++, moderate ++, light +). For the α_2 oligonucleotide (Figs. 1, 2), labeled cells were identified within the MPN (+++), median preoptic nucleus (+++), anteroventral periventricular nucleus (++), supraoptic nucleus (+++), horizontal nucleus of the diagonal band of Broca (++), and suprachiasmatic nucleus (+) of the anterior hypothalamic/POA region; the PrN-BNST (+++), BNAC (+++), magnocellular and posterodorsal nuclei (+++), and anterolateral division (++) of the BNST (classification of Ju and Swanson; 1989); lateral septum (+++), piriform cortex (+++), cingulate cortex layers 2 and 3 (+++), cerebral cortex (elsewhere) layers 2 and 3 (++), and caudate-putamen (+). The distribution of the β_3 signal was remarkably similar to that observed for α_2 being found in all of the regions described above, although overall numbers of β_3-derived silver grains were lower than for α_2 (Figs. 2, 3). Moderate or heavy hybridization signal for γ_1 (Fig. 2) was restricted to the hypothalamic/POA/BNST regions; MPN (+++), anteroventral periventricular nucleus (++), PrN-BNST (+++), BNAC (++), magnocellular and posterodorsal nuclei (+++), and anterolateral division of the BNST (++). At the exposure times used in these experiments γ_1-expressing cells were not detected in the cerebral cortex including piriform and cingulate regions. Competition experiments with unlabeled oligonucleotides abolished specific labeling of cells with all three oligonucleotides (Fig. 2).

Quantitative silver grain analysis was carried out in the MPN,

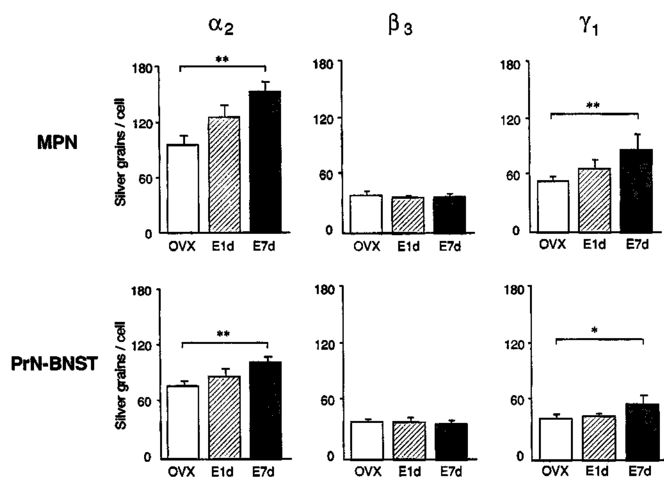

Figure 3. Quantitative analysis of silver grain numbers per cells following α_2, β_3, and γ_1 subunit mRNA hybridizations in the medial preoptic nucleus (*MPN*) and principle encapsulated nucleus of the bed nucleus of the stria terminalis (*PrN-BNST*) of ovariectomized rats treated with oil (*OVX*) or estradiol for 1 (*Ed1*) or 7 (*E7d*) d. Bars give means + SEM. $^*p < 0.05$, $^{**}p < 0.01$.

PrN-BNST, and BNAC for all three oligonucleotides and in the cingulate cortex (layers 2 and 3) for α_2 and β_3. Estrogen treatment for 7 d (OVX-E7d) was found to significantly increase the numbers of silver grains/cell in the MPN and PrN-BNST for the α_2 ($p < 0.01$–0.05) and γ_1 ($p < 0.01$–0.05) but not the β_3 subunit (Fig. 3). In each of these regions, OVX-E1d rats showed an intermediate number of silver grains/cell compared with the other two groups, but were not significantly different from either ($p > 0.05$). No significant differences were detected for α_2, β_3, or γ_1 subunits in the BNAC or α_2 and β_3 subunits in the cingulate cortex (Table 1). The numbers of cells expressing α_2, β_3, or γ_1 mRNA were not quantified, but it appeared that almost all cells in the MPN and PrN-BNST expressed α_2 and γ_1 subunits while around two-thirds of these cells expressed the β_3 subunit (Fig. 2). The three subunits were expressed by all cells in the BNAC.

Immunocytochemistry experiments

Immunoreactivity for the $GABA_A$ receptor α_2 subunit was detected throughout the coronal brain sections as either a diffuse neuropil or distinct cell body staining. As reported previously with this antibody (Zimprich et al., 1991), immunoreactivity localized to cell bodies existed as a combination of cytoplasmic and membrane staining. Within the rostral hypothalamic area, strongly immunoreactive cells were detected in the MPN (Figs. 4*A*,*B*; 5*A*), anteroventral periventricular and supraoptic nuclei, while less intense immunoreactivity was observed in the suprachiasmatic nucleus and lateral hypothalamus. In the septum and BNST, strong immunoreactivity was restricted to cell body staining in the lateral septum and BNAC (Fig. 4*C*), with the PrN-BNST (Fig. 5*D*), magnocellular and posterodorsal nuclei, and anterolateral division of the BNST exhibiting moderate to light cell body staining. Elsewhere in the coronal sections, strong to moderate immunoreactivity was observed within cells in the piriform and cingulate (Fig. 4*D*) cortices and the horizontal nucleus of the diagonal band of Broca.

Estrogen receptor staining was identical to that reported by others (Cintra et al., 1986) and ourselves (Herbison and Theodosis, 1992) in these areas and consisted of nuclear labeling of large numbers of cells in the MPN (Fig. 5*B*), PrN-BNST (Fig. 5*E*) and posterodorsal nucleus of the BNST. ER-immunoreactive cells were also noted outside the MPN in the periventricular region, lateral hypothalamus, and throughout the BNST including the magnocellular and anterolateral nuclei but not the BNAC. As first noted by Cintra and colleagues (1986), nonspecific staining of ependymal cells was also evident.

Double labeling experiments revealed large numbers of cells exhibiting black ER nuclear staining combined with brown α_2 cytoplasmic/membrane staining in the MPN (Fig. 5*C*). Smaller numbers of lighter stained double-labeled cells were detected in the PrN-BNST (Fig. 5*E*, inset). Cell count estimates showed that 67 ± 3% of the α_2 subunit immunoreactive cells in the MPN possess ERs and that these double-labeled cells accounted for 17 ± 2% of ER-containing cells in the MPN. As α_2 subunit immunoreactivity was relatively faint in the BNST after the double-labeling procedure, a semiquantitative analysis of single- and double-labeled cells was not undertaken.

Discussion

The present results show that circulating estrogen concentrations influence mRNA levels of specific $GABA_A$ receptor subunits within ER-containing regions of the rat brain. Further, we show that the α_2 subunit is expressed by cells possessing ERs in the MPN and PrN-BNST. Such findings indicate that estrogen may alter gene transcription to influence $GABA_A$ receptor functioning in specific regions of the rat brain. In particular, it is important to note that the MPN is established as an important brain structure in the gonadal steroid-dependent control of reproductive behavior and reproductive hormone secretion in female rats (Freeman, 1994; Pfaff et al., 1994). Although the BNST is sim-

Table 1. Silver grain analysis of α_2, β_3, and γ_1 subunit mRNA expression in the layers 2 and 3 of the cingulate cortex and bed nucleus of the anterior commissure in ovariectomized (OVX) rats and following estrogen treatment for 1 d (OVX-E1d) or 7 d (OVX-E7d).

Area	Treatment	α_2	β_3	γ_1
Cingulate cortex	OVX	88.3 ± 6.9	62.9 ± 4.5	—
(silver grains/cell)	E1d	82.8 ± 5.0	49.7 ± 4.8	—
	E7d	77.7 ± 2.5	60.0 ± 7.9	—
Bed nucleus of the anterior	OVX	0.89 ± 0.17	0.28 ± 0.03	0.23 ± 0.01
commissure (silver grains/	E1d	0.74 ± 0.12	0.28 ± 0.05	0.24 ± 0.02
μm^2	E7d	1.15 ± 0.28	0.30 ± 0.03	0.26 ± 0.03

For the cingulate cortex, numbers give mean ± SEM for number of silver grains/cell while analysis in the bed nucleus of the anterior commisure gives silver grain densities for the whole nucleus. Note that γ_1 mRNA-expressing cells were not detected in the cingulate cortex.

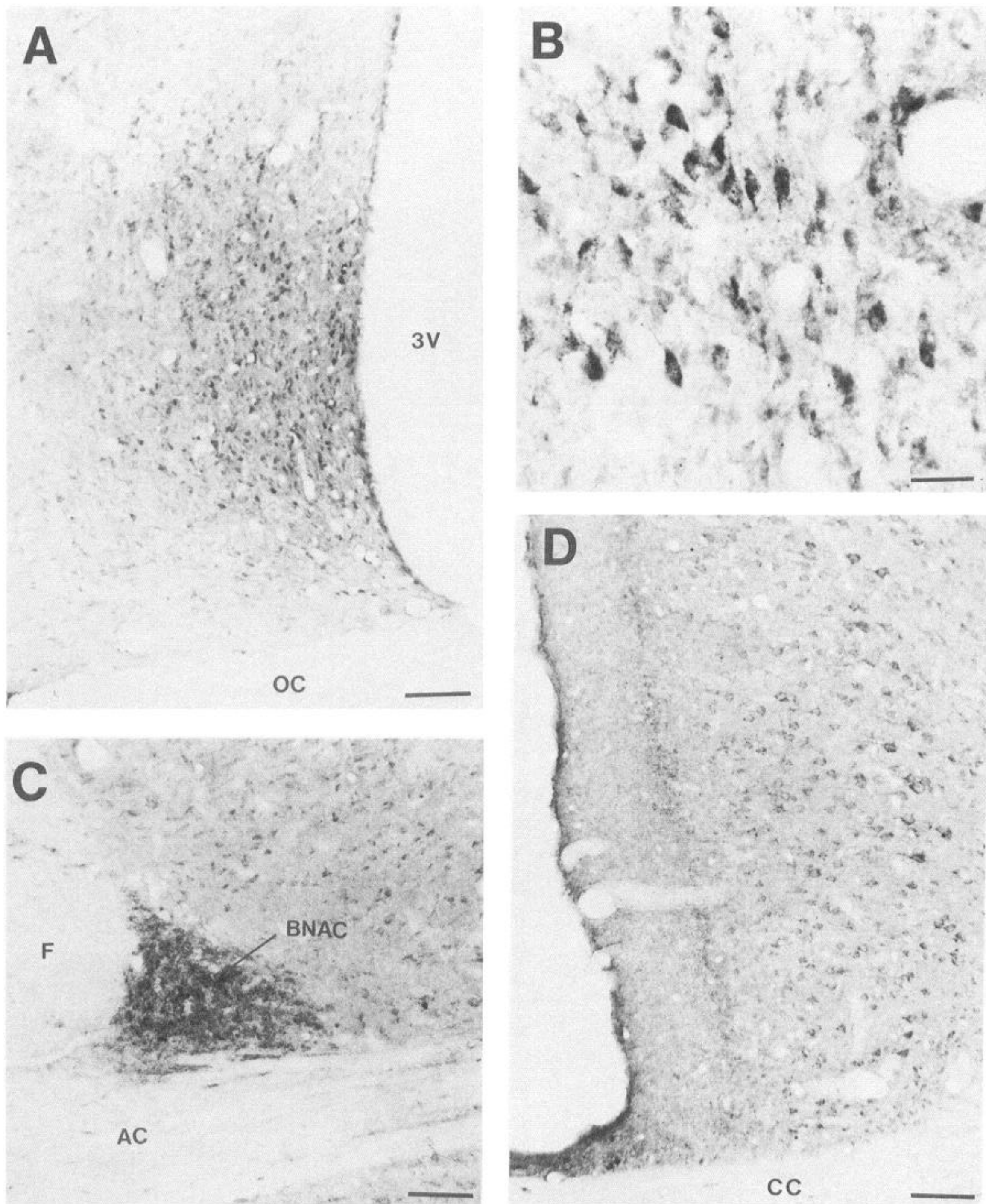

Figure 4. Immunocytochemical staining for the α_2 subunit of the $GABA_A$ receptor in the medial preoptic nucleus (*A* and *B*), bed nucleus of the anterior commissure (*C*), and cingulate cortex (*D*). *AC,* anterior commissure; *BNAC,* bed nucleus of the anterior commissure; *CC,* corpus callosum; *F,* fornix; *OC,* optic chiasm; *3V,* third ventricle. Scale bars: *A* and *C*, 80 μm; *B*, 20 μm; *D*, 50 μm.

ilarly implicated in the neural control of reproduction (Terasawa and Kawakami, 1974), the precise roles of its various subnuclei are not well established. Nevertheless, the selective predominance of ER-containing neurons in the PrN-BNST and the strong Fos expression in this region following mating in female rats (Tetel et al., 1993) suggest a role in the integration of steroidal and sensory stimuli with respect to reproductive behavior.

The distribution of hybridized cells and relative strength of expression for α_2, β_3, and γ_1 mRNA transcripts in our experiments appear identical to that reported by Wisden and colleagues (1992) using the same ^{35}S-labeled oligonucleotide probe sequences and are in good agreement with other workers examining α_2 (MacLennan et al., 1991), β_3 (Zhang et al., 1991), and γ_1 (Ymer et al., 1990) mRNA expression in the rat brain. With respect to the α_2 subunit, it is reassuring to note that the distribution of α_2 mRNA-positive cells is in good agreement with our α_2 immunoreactivity observations. In particular, the strong expression of α_2 mRNA in the MPN and

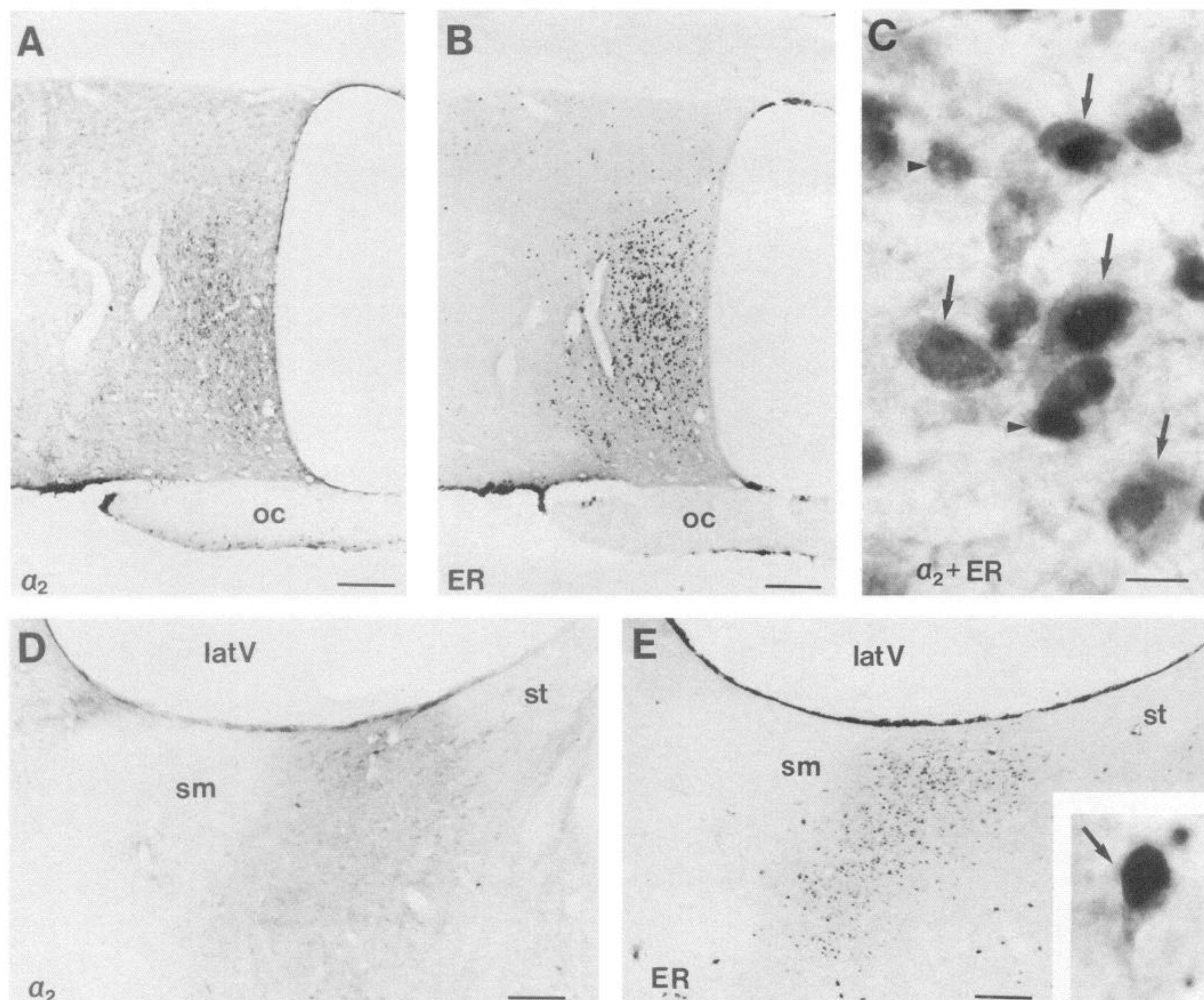

Figure 5. Single- and double-labeling immunocytochemistry stainings on consecutive sections for the α_2 subunit of the $GABA_A$ receptor (*A* and *D*), estrogen receptor (*ER*; *B* and *E*), and both receptors (*C* and *E, inset*) at the level of the medial preoptic nucleus (*A–C*) and principle encapsulated nucleus of the bed nucleus of the stria terminalis (*D* and *E*). Note the overlapping distribution of the α_2 subunit- and estrogen receptor immunoreactivity in the medial preoptic nucleus (*A* and *B*). *C* shows a high-power view of double-labeled cells (*arrows*) immunoreactive for the estrogen receptor (black nuclear stain) and α_2 subunit (cytoplasmic/membrane immunoreactivity). Cells only immunoreactive for the estrogen receptor are indicated by *arrowheads*. In the principle encapsulated nuclei note the relatively faint α_2 subunit staining (*D*) that overlaps with that of the estrogen receptor distribution (*E*) and that double-labeled cells were also detected (*arrow, inset, E*). *ER*, estrogen receptor; *latV*, lateral ventricle; *sm*, stria medularis; *st*, stria terminalis; *oc*, optic chiasm. Scale bars: *A* and *B*, 200 μm; *C*, 10 μm; *D* and *E*, 150 μm.

BNAC correlates well with the intense α_2 immunoreactivity in these regions. While this correlation is good at a qualitative level, as it is elsewhere in the brain for α_2 mRNA and this particular antibody (Zimprich et al., 1991; Wisden et al., 1992), the relationship, in general, between $GABA_A$ receptor protein levels and mRNA expression is not well defined. We find here that all of the regions expressing strong α_2 subunit immunoreactivity such as the MPN, median preoptic nucleus, lateral septum, cingulate, and piriform cortices, also have the highest levels of α_2 subunit mRNA expression. However, we note that silver grain numbers per cell in the PrN-BNST are not substantially different from those of MPN cells in the ovariectomized rat and yet α_2 subunit immunocytochemistry is noticeably weaker in the former. Furthermore, our *in situ* data suggest that the great majority of MPN cells synthesize α_2 subunit mRNAs and yet our immunocytochemical results show that it is only a subpopulation of MPN cells, possibly half, that possess detectable immunoreactivity. Such results may depend solely on the limitations of immunocytochemical analysis at the level of the light microscope. Nevertheless, the differences in α_2 immunoreactivity between two regions expressing approximately equivalent amounts of mRNA raises the issues of posttranslational modification of $GABA_A$ receptor proteins and the possible dependence of α_2 immunoreactivity on its subunit glycosylation and/or association with other subunits within the hetero-oligomer.

As noted previously by Wisden and colleagues (1992), there is substantial overlap in the distribution of α_2 and β_3 subunit

mRNAs throughout the brain. However, as mRNA for the γ_1 subunit is only readily detected in the POA, hypothalamus, and BNST, the combined expression of α_2, β_3, and γ_1 mRNAs is restricted almost entirely to these hypothalamic/limbic regions of the brain (Ymer et al., 1990; Wisden et al., 1992). In this study, we observed that the great majority of cells in the MPN, PrN-BNST, and BNAC express α_2, β_3, and γ_1 subunit mRNAs. This suggests that individual cells in these areas may synthesize all three subunits and that an $\alpha_2\beta_3\gamma_1$ isoform of the GABA$_A$ receptor may be used by these cells. Although we are unable to verify this hypothesis from the present studies, the apparent absence of abundant mRNA transcripts for other subunits of the GABA$_A$ receptor within the MPN and PrN-BNST (Zhang et al., 1991; Wisden et al., 1992) strengthens this possibility. If this is the case, the $\alpha_2\beta_3\gamma_1$ isoform of the GABA$_A$ receptor, which may also exist in the medial amygdala (see Wisden et al., 1992), would provide a selective pharmacological target for limbic and neuroendocrine networks in the brain. To our knowledge, the pharmacology of an expressed $\alpha_2\beta_3\gamma_1$ GABA$_A$ receptor isoform has not been examined, although extracellular recordings *in vivo* have shown inhibitory responses from almost all cells in the POA in response to GABA (Mayer, 1981).

Estrogen has been reported previously to alter ^{35}S-TBPS binding in the POA of the rat (Canonaco et al., 1993). We show here that estrogen increases mRNA expression of α_2 and γ_1, but not β_3, subunits within this region. It is known that muscimol binds relatively weakly to GABA$_A$ receptors possessing α_2 subunits (McKernan et al., 1991) and the observation here of abundant, estrogen-regulated α_2 mRNA expression may underlie both the poor labeling of the POA and BNST with ^{3}H-muscimol (Olsen et al., 1990) and the inability of a variety of estrogen treatments to alter muscimol labeling in these regions (O'Connor et al., 1988). In contrast, TBPS binding is strong in this area (Olsen et al., 1990) and reduced following the administration of estrogen to ovariectomized rats for 24 hr (Canonaco et al., 1993). Although the precise manner of TBPS binding to the GABA$_A$ receptor is unknown, the inverse correlation between estrogen effects on α_2 and γ_1 mRNA expression and TBPS binding suggests that this ligand may have relatively low affinity for one or both of these subunits.

In present study, significant differences in α_2 and γ_1 subunit mRNA expression were detected after treating ovariectomized animals with estrogen for 7 d; animals given estrogen for 24 hr always showed increased α_2 and γ_1 expression in the MPN and PrN-BNST, although this was not significantly different to levels observed in control animals. The increase in plasma estradiol concentrations from low levels in ovariectomized rats to approximately 25 pg/ml in our estrogen-treated animals (Leipheimer et al., 1984) are well within the range of estradiol concentration fluctuations of 10–50 pg/ml seen during the estrous cycle in the rat (Freeman, 1994). The specificity of this selective increase in GABA$_A$ receptor subunit mRNA expression for brain regions possessing the ER is demonstrated by the lack of any change in the cingulate cortex and, in particular, cells of the BNAC that synthesize $\alpha_2\beta_3\gamma_1$ subunits, but unlike their near neighbors in the BNST, do not possess ERs.

We are unable to determine from this study whether the mRNA changes following estrogen treatment result in alterations of existing GABA$_A$ receptor subunit composition or an increase in the number of GABA$_A$ receptors possessing α_2 and γ_1 subunits. As it is believed that each GABA$_A$ receptor contains only one α subunit type (Duggan and Stephenson, 1990; McKernan et al., 1991), the increase in α_2 mRNA observed here following estrogen may represent the inclusion of more α_2 subunits within each receptor hetero-oligomer, or equally more GABA$_A$ receptors with an equivalent α_2 subunit composition. It appears likely that one or multiple β subunits are essential components of all GABA$_A$ receptors (Ymer et al., 1989; Sigel et al., 1990; Wisden et al., 1992; Amin and Weiss, 1993) and that the β_3 subunit may predominate within the MPN and BNST (Zhang et al., 1991; Wisden et al., 1992). Hence, it may be of significance that β_3 subunit mRNA is not altered by estrogen, suggesting that the number of GABA$_A$ receptors utilizing the β_3 subunit remain stable and that a change of α and γ subunits within a β_3-containing GABA$_A$ receptor may occur. In preliminary observations, Petersen and colleagues (1993b) have similarly reported that estrogen treatment does not alter β_2 or β_3 subunit mRNA expression in the region of the organum vasculosum of the lamina terminalis.

Evidence for the regulation of GABA$_A$ receptors by estrogen reported here adds a further dimension to our understanding of estrogen–GABA interactions in the POA. Previous experiments have demonstrated that the local preoptic GABA neurons possess ERs (Flügge et al., 1986) and that extracellular GABA concentrations as measured *in vivo* by push–pull (Ondo et al., 1982) and microdialysis (Herbison et al., 1990, 1991a) are elevated in the presence of estrogen. Indirect evidence suggests that this effect of estrogen on GABA neurons is exerted through the genome (Herbison et al., 1991a) but that it does not involve changes in the activity (O'Connor et al., 1988) or mRNA expression (Herbison et al., 1992) of glutamic acid decarboxylase$_{67}$, the principle GABA synthetic enzyme. Estrogen has also been shown to modulate the stimulatory α-adrenergic receptor-mediated input to GABA neurons in the POA (Herbison et al., 1990). Such effects of estrogen are likely to have important consequences for the neural control of behavior and reproduction as sexual behavior (Fernandez-Guasti et al., 1985; McCarthy et al., 1991) and locomotor activity (Osborne et al., 1993) as well as prolactin and luteinizing hormone secretion (Lamberts et al., 1983; Herbison et al., 1991b; Herbison and Dyer, 1991) are all altered by pharmacological manipulations of the preoptic GABA network. The demonstration here that estrogen can also alter GABA$_A$ receptor subunit mRNA expression in the POA indicates the complex and multifaceted influence of estrogen on neural networks involving GABA neurons in the POA of the rat.

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