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MATERNAL BEHAVIOUR IN LACTATING RATS STIMULATES c-*fos* IN GLUTAMATE DECARBOXYLASE-SYNTHESIZING NEURONS OF THE MEDIAL PREOPTIC AREA, VENTRAL BED NUCLEUS OF THE STRIA TERMINALIS, AND VENTROCAUDAL PERIAQUEDUCTAL GRAY

J. S. LONSTEIN* and G. J. DE VRIES

Center for Neuroendocrine Studies, Tobin Hall, Box 37720, University of Massachusetts, Amherst, MA 01003, USA

Abstract—Increased activity of the immediate-early gene c-*fos* can be observed in many areas of the lactating rat brain after dams physically interact with pups and display maternal behaviour. These sites include the medial preoptic area, ventral bed nucleus of the stria terminalis, and the ventrolateral caudal periaqueductal gray, each of which is critical for the normal performance of particular maternal behaviours. The phenotype of cells in these areas that show increased c-*fos* activity after maternal behaviour, however, is unknown. Via double-label immunocytochemistry, we determined if the population of cells in these sites that express c-*fos* after maternal behaviour in lactating rats overlaps with the population that expresses the 67,000 mol. wt isoform of glutamate decarboxylase, the synthesizing enzyme for the inhibitory neurotransmitter GABA. Lactating rats were separated from pups beginning on day 5 postpartum, and 48 h later half were allowed to interact with a litter of pups for 60 min whereas the other half were not. Dams re-exposed to pups were highly maternal, retrieving and licking them as well as displaying prolonged nursing behaviour that included milk letdown. Both groups of dams had a similar number of 67,000 mol. wt glutamate decarboxylase-immunoreactive cells in each site, although the number of 67,000 mol. wt glutamate decarboxylase-immunoreactive cells per microscopic field was significantly greater in the caudal ventrolateral periaqueductal gray than in the ventral bed nucleus of the stria terminalis, which in turn was greater than the medial preoptic area. In pup-stimulated dams, two to fourfold more Fos-immunoreactive cells were found in these three sites compared with non-stimulated controls. Labeling for Fos immunoreactivity and 67,000 mol. wt glutamate decarboxylase immunoreactivity was heterogeneous within each site. In the medial preoptic area, more Fos-immunoreactive and 67,000 mol. wt glutamate decarboxylase-immunoreactive cells (either single or dual-labeled) were found dorsally than ventrally. In the ventral bed nucleus of the stria terminalis, more Fos-immunoreactive and 67,000 mol. wt glutamate decarboxylase-immunoreactive cells were found medially than laterally. Within the caudal ventrolateral periaqueductal gray, 67,000 mol. wt glutamate decarboxylase-immunoreactive labeling was greatest ventromedially, while high numbers of Fos-immunoreactive nuclei were found both ventromedially and ventrolaterally. In pup-stimulated dams, more than half (53% in the medial preoptic area, 59% in the ventral bed nucleus of the stria terminalis, and 61% in the caudal ventrolateral periaqueductal gray) of the total population of Fos-immunoreactive cells also expressed 67,000 mol. wt glutamate decarboxylase.

These results suggest that many of the neurons in these sites that show elevated c-*fos* activity after maternal behaviour are either local inhibitory interneurons or provide inhibitory input to other neural sites. These inhibitory mechanisms may be critical for the display of postpartum nurturance, possibly facilitating maternal behaviour by removing tonic inhibition on sites necessary for maternal responding or by restricting activity in neural sites that inhibit it.

Key words: disinhibition, immediate-early gene, GABA, milk ejection, nursing behaviour, somatosensation.

Maternal behaviour in rats is a complex aggregation of activities that rely on different somatosensory inputs and neural structures for their control.[71,94,95,100] Although many areas of the brain may act collectively to produce the coordinated display of maternal behaviour, three neural sites are crucial for specific components of ongoing maternal care in postpartum rats. The medial preoptic area (mPOA) and adjacent ventral bed nucleus of the stria terminalis (vBST) are critical for particular active maternal behaviours carried out with the mouth, such as retrieval, that depend on stimulation of the trigeminal nerve for their display.[71,74,96,98,99] In contrast, the lateral and ventrolateral regions of the midbrain periaqueductal gray ($cPAG_{l,vl}$) are necessary for quiescent nursing behaviour in the typical kyphotic (upright crouched) posture,[52,54,55,85] which requires sufficient suckling by the pups.[56,97,102]

Several studies using immunocytochemical visualization of c-*fos* activity as a marker for neuronal modulation have supported a role for the mPOA and vBST in the performance of retrieval and the $cPAG_{l,vl}$ in the display of kyphosis. Significant increases in the Fos protein product of c-*fos* can be found in the mPOA and vBST after dams retrieve pups and perform other oral maternal behaviours.[24,57,72,73,75,77,112] Levels of Fos labeling in these sites are not affected by the presence or absence of suckling.[57,73,112] Alternatively, suckling by the pups and subsequent nursing by the dam are necessary for high levels of c-*fos* activity in the $cPAG_{l,vl}$ of dams that interact with pups.[52,53] Furthermore, the number of Fos-immunoreactive (Fos-IR) cells in the $cPAG_{l,vl}$ are positively correlated with the duration of kyphosis displayed by the dam and are

*To whom correspondence should be addressed. Tel.: +1-413-545-0526; fax: +1-413-545-0996.

E-mail address: lonstein@cns.umass.edu (J. S. Lonstein).

Abbreviations: ANOVA, analysis of variance; $cPAG_{vl}$, caudal ventrolateral periaqueductal gray; $cPAG_{l,vl}$, caudal lateral and ventrolateral periaqueductal gray; ERα, alpha form of the estrogen receptor; Fos-IR, Fos-immunoreactive; GAD, glutamate decarboxylase; GAD_{65}, 65,000 mol. wt isoform of glutamate decarboxylase; GAD_{67}, 67,000 mol. wt isoform of glutamate decarboxylase; GAD_{67}-IR, 67,000 mol. wt glutamate decarboxylase-immunoreactive; MPN, medial preoptic nucleus; PAG, periaqueductal gray; mPOA, medial preoptic area; PBS, phosphate-buffered saline; TBS, Trisma-buffered saline; vBST, ventral bed nucleus of the stria terminalis; VMH, ventromedial nucleus of the hypothalamus.

not significantly affected by the dam's performance of oral maternal activities.[53]

It is difficult to speculate how neurons that show increased c-*fos* activity after the performance of maternal behaviours contribute to the display of these behaviours because the phenotype of these cells has not been determined. Given that between 50–95% of neurons in the mPOA and vBST, and 30–50% of neurons in the $cPAG_{l,vl,}$ synthesize the inhibitory neurotransmitter GABA,[2,69] it may be that many Fos-IR neurons in these sites are GABAergic. Activity of glutamate decarboxlyase (GAD), the rate-limiting synthesizing enzyme for GABA, can be influenced by ovarian hormones in many neural sites, including the mPOA and periaqueductal gray (PAG),[26,39,63,84,86,111] and it is conceivable that the hormones associated with gestation or lactation are tightly linked with GAD activity in the lactating rat brain. Indeed, many changes in GABAergic activity are found in the brain of postpartum animals,[9,18,19,30,35,46,70,81,109] which may be important for a multitude of functions including milk production and let-down,[19,30,109] reduced hypothalamic-pituitary-adrenal activity in response to stress,[101] suckling-induced slow-wave sleep,[110] inhibition of gonadotropin secretion,[108] hyperphagia,[21] as well as maternal behaviour.

We examined this possibility by determining the extent of overlap between the populations of cells in the mPOA, vBST, and caudal ventrolateral periaqueductal gray ($cPAG_{vl}$) of lactating rats that express increased c-*fos* activity after the performance of maternal behaviour and those that express the 67,000 mol. wt isoform of GAD (GAD_{67}). We immunocytochemically detected GAD_{67} because, unlike the smaller 65,000 mol. wt isoform of GAD (GAD_{65}) that is found primarily in nerve terminals, the larger GAD isoform can be found within the cytoplasm of neuronal somata[60] and therefore can be clearly co-localized with the Fos protein, which is primarily found in the nucleus.[13] Although GAD_{65} may be the isoform that is particularly important for extracellular GABA release,[60] the vast majority of neurons that express GAD_{65} in their terminals also express GAD_{67} in their somata.[91,103]

EXPERIMENTAL PROCEDURES

Subjects

Subjects were 19 Sprague–Dawley female rats (Taconic, Germantown, New York) purchased at 65–75 days-old and mated with sexually experienced Sprague–Dawley males from our colony one week after arrival. Females were housed in groups of two to three animals in wire hanging cages. Three to four days prior to the expected day of parturition, subjects were individually housed in clear polypropylene cages ($48 \times 28 \times 16$ cm) with wood shavings for bedding. Dams were then placed in a small colony room containing pregnant females and lactating dams with their litters for the remainder of the experiment. Dams were completely undisturbed during the 48 h separation from pups. Food and water were available *ad libitum*, lights were on between 0800–1600 daily, and the ambient temperature was $\sim 22 \pm 1$°C. Litters were culled to contain eight pups (four males and four females) within 24 h after parturition. During the 48-h mother–litter separations, litters were given to surrogate lactating dams from our colony of the same lactational stage as the biological mother. All efforts were made to minimize both the suffering and number of animals used. All experimental procedures conformed to the University of Massachusetts and National Institutes of Health guidelines on the ethical use of animals in research.

Behavioural testing

On the morning of day 5 postpartum, dams had their litters removed and were rehoused in clean clear polypropylene pan cages with clean bedding. Forty-eight hours later, dams were either exposed to pups (pup-stimulated, $n = 10$) or not (non-stimulated, $n = 9$). For dams that were exposed to pups, seven-day-old litters were removed from surrogate lactating dams between 0900–1030 and incubated at nest temperature (~34°C) in a paper-lined glass bowl for 3 h prior to behavioural testing. After the 3-h of incubation, pups were expressed of feces and urine and weighed. Litters were then scattered in the home cage diagonally opposite to where the dam was sitting. Mother–litter interactions were continuously observed for 60 min as described previously[52] with the aid of a computerized data acquisition system that provided information on behavioural frequencies, latencies, and durations. Behaviours recorded included retrieval of the pups into the nest, full-body and anogenital licking of the pups, self-grooming, exploration, nesting/burrowing in the wood shaving bedding, as well as hovering over the pups in the nest (a non-nursing position) while actively performing other behaviours such as licking of pups or self-grooming, and three mutually-exclusive quiescent 'nursing' postures that females were observed to display over pups: kyphosis,[95] or upright crouching over the litter in a high or low-arched posture,[97] laying prone on top of the litter mass with little or no limb support, and sitting hunched over the litter with the body weight primarily resting on the hind limbs and hind flanks with the forelimbs often passively resting on the litter mass.[52] Pup stretch responses to milk receipt[16] were also recorded. After the behavioural observation, pups were immediately removed from the dam's cage and weighed. Non-stimulated dams had their cage tops briefly removed and replaced as if pups were being introduced, and again 60 min later as if pups were being removed. All subjects remained alone in their home cage for another 60 min, after which they were deeply anesthetized with an overdose of pentobarbital (Sigma, USA).

Immunocytochemistry

Anesthetized dams were perfused through the heart with 150 ml of 0.9% saline followed by 150 ml of 4% paraformaldehyde (Sigma, USA) dissolved in 0.1 M sodium phosphate-buffered saline (PBS, pH 7.4). Brains were removed and postfixed overnight in 4% paraformaldehyde in PBS and submerged in 30% sucrose in PBS for at least three days before sectioning. Within five days after perfusion, entire brains were cut on a freezing microtome into 35 μm coronal sections, which were stored in a PBS-buffered cryoprotectant (pH 7.4) until immunocytochemical processing.

Every fourth section through the brain was processed immunocytochemically for Fos-IR and 67,000 mol. wt glutamate decarboxylase-immunoreactive (GAD_{67}-IR) cells and sections from all subjects were included in one immunocytochemical run. Our data obtained from alternate brain sections from these subjects that were labeled for the Fos and estrogen receptor alpha (ERα) proteins can be found elsewhere.[58] Free-floating sections were washed three times in 0.5 M Trisma-buffered saline (TBS, pH 7.6) for 5 min each rinse, incubated for 15 min in 0.5% sodium borohydride, washed three times in TBS, incubated for 20 min in 1.5% hydrogen peroxide, rinsed three times with TBS, incubated for 45 min in 20% normal goat serum in 0.03% Triton X-100 in TBS, and then incubated overnight (~18 h) at 22°C in a solution of 0.5 M TBS with 2% goat serum and 0.03% Triton X-100 containing a rabbit polyclonal anti-c-*fos* antiserum that recognizes residues 4–17 of the human Fos protein (Ab-5, 1:2000; Oncogene Science, Manhassat, New York). Although we typically use 0.03% Triton X-100 to enhance penetration of the antibodies during immunocytochemistry in our laboratory, the manufacturer's recommended protocol provided with the GAD_{67} primary antiserum indicated that Triton X-100 reduced GAD_{67} immunostaining. In pilot experiments, however, we found that concentrations of Triton X-100 only greater than 0.15% reduced GAD_{67} immunostaining whereas concentrations of Triton X-100 greater than 0.03% did not further enhance Fos immunostaining. After incubation in the Fos primary antiserum, sections were rinsed three times in TBS, incubated for 60 min in a solution of 2% normal goat serum in TBS and a biotinylated goat anti-rabbit secondary antiserum (Vector Labs, Burlingame, CA, USA). After rinsing three times in TBS, sections were incubated for 60 min in avidin–biotin complex (Vectastain Elite; Vector Labs), rinsed three times, and incubated for ~10 min in 0.05% 3′-3-diaminobenzadine with 1.5% nickel ammonium sulfate, 0.04% ammonium chloride, 0.0004% glucose oxidase, and 0.15% -D-glucose in TBS, which provided a dark purple or black nuclear stain. Sections were rinsed five times in TBS and then run for GAD_{67} immunocytochemistry with a rabbit primary polyclonal antiserum that recognizes GAD_{67} (AB108,

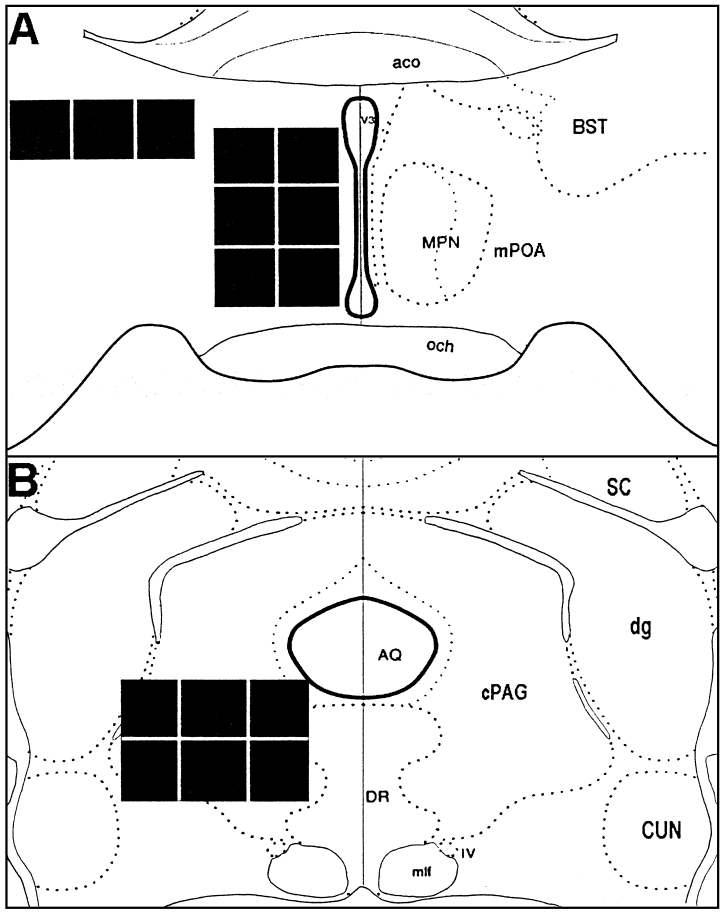

Fig. 1. Diagrammatic representation of the areas of the (A) mPOA and vBST, and (B) $cPAG_{vl}$ analysed for Fos-IR and GAD_{67}-IR labeling. Black boxes represent the three (vBST) or six (mPOA, $cPAG_{vl}$) adjacent square microscopic areas analysed. Aco, anterior commisure; AQ, cerebral aqueduct; cPAG, caudal periaqueductal gray; CUN, cuneiform nucleus; dg, deep gray layers of superior colliculus; DR, dorsal raphe; mlf, medial longitudinal fasciculus; mPOA, medial preoptic area; MPN, medial preoptic nucleus; och, optic chaism. SC, superior colliculus; vBST, ventral bed nucleus of the stria terminalis; IV, trochlear nucleus. Modified from Ref. 105.

1:4000; Chemicon, Temecula, CA, USA). The GAD_{67} primary antibody was used in 2% normal goat serum in TBS; no Triton X-100 was added. After an overnight incubation at 4°C, sections were processed with secondary antiserum and avidin-biotin complex as described above. Sections were rinsed in TBS and incubated in 0.05% 3-3′-diaminobenzadine with 0.0006% hydrogen peroxide for ~20 min, which provided a light brown cytoplasmic staining for GAD_{67}. Sections were rinsed, mounted onto microscope slides, dehydrated, and coverslipped. Controls for immunocytochemical specificity included sections incubated with only one or neither primary antiserum, which produced only single labeling or eliminated both Fos-IR and GAD_{67}-IR labeling, respectively.

Microscopic analysis

Slides were randomized and coded for microscopic analysis so that the group designation of subjects was unknown during analysis. The number of cells within the mPOA, vBST, and $cPAG_{vl}$ that showed Fos-IR, GAD_{67}-IR, and Fos-IR plus GAD_{67}-IR labeling were quantified by eye with the aid of a reticle placed in one ocular lens. Fos-IR labeling was evident by dark purple or black staining restricted to the cell nucleus and GAD_{67}-IR labeling was identifiable by light brown staining throughout the cytoplasm of cell. Dual-labeled neurons were identifiable by the presence of a dark purple or black Fos-IR nucleus surrounded by a light brown 'halo' of cytoplasmic staining for GAD_{67}. No black cytoplasmic staining or light brown nuclear staining was ever observed. Immunostaining in the mPOA and vBST was analysed at the level at which the most Fos-IR neurons are found after maternal behaviour in lactating rats,[72,73] at approximately −0.46 mm from bregma, most closely corresponding to plate #20 from Swanson's[105] atlas of the adult male rat brain (Fig. 1a). Although morphological differences may exist between some subregions of the mPOA and vBST of male and female rats,[15,31] the atlas of the male rat brain is of considerable heuristic value and we will describe our neural sites analysed in reference to the same areas indicated for the male rat brain. The dorsal third of the area analysed within the mPOA included the anterodorsal preoptic nucleus and the most dorsal part of the medial preoptic nucleus (MPN). The middle third of the mPOA area analysed included almost the entire dorsal half of the MPN, encompassing the entire central nucleus and dorsal areas of the medial and lateral nuclei. The ventral third of the mPOA area analysed included the ventral MPN (ventral medial and lateral nuclei). The anteroventral and periventricular preoptic nuclei were outside our areas of analysis. The medial area of analysis in the vBST primarily included the anterior magnocellular division, as well as the medial ventral nucleus and a small part of the lateral dorsomedial nucleus. The middle part of the vBST analysed included the anteroventral and ventral nuclei. The most lateral area of the vBST analysed primarily included the anterolateral division and the ventral rhomboid nucleus. Immunostaining in the $cPAG_{vl}$ was analysed at the level of the PAG where suckling induces high levels of c-*fos* activity,[52,53] which is at approximately −7.6 mm from bregma and corresponds to plate #44 from Swanson[105] (Fig. 1b). The ventral half of the $cPAG_{vl}$ analysed included the most ventral region of the ventrolateral functional column, as described by Bandler *et al.*[1] The dorsal half of the $cPAG_{vl}$ analysed included the dorsal part of the ventrolateral functional column as well as the ventral portion of the lateral functional column.

Immunoreactive labeling was visualized at ×40 with a Nikon Optiphot 2 microscope using a blue filter. Square-shaped microscopic areas (280 × 280 μm) were analysed and the number of single and dual-labeled neurons were directly quantified by a single observer (J.S.L.). The range of Fos-IR and GAD_{67}-IR intensities was small

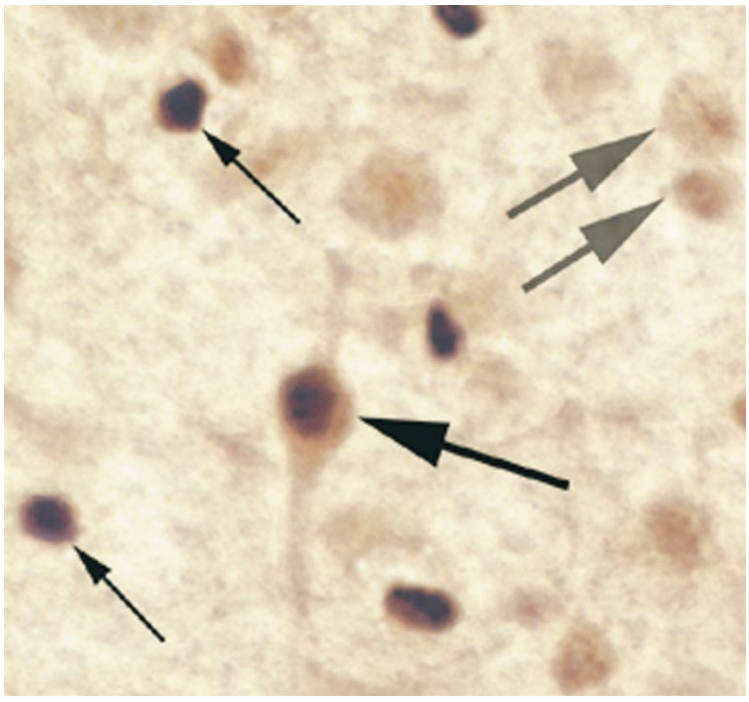

Fig. 2. Photomicrograph of GAD_{67}-IR (large gray arrows), Fos-IR (small black arrows), and dual-labeled (large black arrow) neurons in a partial microscopic field of analysis within the $cPAG_{vl}$ of a pup-stimulated lactating rat. Magnification at × 40.

and all neurons containing any Fos or GAD_{67} immunoreactivity were quantified. Each subject had one section per site chosen for analysis and each section was analysed bilaterally. Sections were chosen by their correspondence to the reference atlas plate and not by the levels or intensity of Fos-IR or GAD_{67}-IR labeling. In the mPOA and $cPAG_{vl}$, six adjacent square microscopic areas were analysed within each hemisphere. In the vBST, three adjacent areas per hemisphere were analysed.

Data analyses

One pup-stimulated dam was poorly perfused and was removed from the study (resulting $n = 9$). Due to poor histology, we were unable to obtain immunocytochemical data from the vBST of one non-stimulated dam (resulting in $n = 8$ for this site). Since no differences between hemispheres in any site were found for Fos or GAD_{67} immunoreactive labeling ($Ps \geq 0.1$), data from each hemisphere were combined for data analyses. Therefore, immunocytochemical data are expressed as the total number of immunoreactive cells quantified in both hemispheres as well as the mean number of immunoreactive cells per square microscopic area. Immunocytochemical data from the mPOA were analysed with a 2 (group) × 2 (mediolateral position) × 3 (dorsoventral position) analysis of variance (ANOVA). Immunocytochemical data from the $cPAG_{vl}$ were analysed with a 2 (group) × 3 (mediolateral position) × 2 (dorsoventral position) ANOVA and data from the vBST were analysed with a 2 (group) × 3 (mediolateral position) ANOVA. Correlations between behavioural and immunocytochemical data for pup-stimulated dams were performed with Pearson's *r* correlation coefficient.

RESULTS

Dam behaviour

The nine pup-stimulated dams included in the study were highly parental after reunion with their litters. The dams retrieved all or most pups (7.2 ± 0.4 pups) to the nest within 3 ± 1 min and spent 54 ± 1 min in physical contact with them. Dams actively hovered over the pups for 24 ± 2 min of this time while licking them (15 ± 2 min) and self-grooming (2 ± 0 min). All dams displayed prolonged periods of quiescent nursing behaviour (total duration = 30 ± 2 min) that included 5 ± 1 pup stretch responses to milk letdown. Periodic spot checks indicated that the non-stimulated dams were generally inactive within a few minutes after removal and replacement of their cage top, as reported previously.[46]

Immunoreactive labeling

All three sites analysed contained many neurons that showed light brown cytoplasmic labeling of GAD_{67} and dark purple or black nuclear labeling of the Fos protein. Dual-labeled neurons were identifiable by the presence of a darkly stained Fos-IR nuclei surrounded by a 'halo' of light brown cytoplasmic GAD_{67}-IR label (Fig. 2). Differences within sites and between groups in the number of labeled neurons and their distribution are detailed below.

Glutamate decarboxylase$_{67}$-immunoreactive labeling

Many neurons containing GAD_{67}-IR labeling were found in all three neural sites investigated. The three sites significantly differed from each other in overall density of GAD_{67}-IR neurons, with the number of GAD_{67}-IR neurons per microscopic field significantly greater in the $cPAG_{vl}$ than in the vBST, both of which were greater than that found in the mPOA ($F_{2,50} = 30.19$, $P \leq 0.0001$; Fig. 3a). Regional differences in GAD_{67}-IR labeling were also found within each site. In the mPOA, there was a significant dorsoventral gradient such that the most GAD_{67}-IR neurons were found in the dorsal third, an intermediate number in the middle third, and the fewest in the ventral third ($F_{1,96} = 16.98$, $P \leq 0.0001$; Table 1, Fig. 4); all regions significantly differed from one another. Furthermore, significantly more GAD_{67}-IR neurons were found medially than laterally ($F_{2,96} = 4.44$, $P \leq 0.04$). There were no significant group effects or interactions effects including group as a variable for the number of GAD_{67}-IR neurons in the mPOA ($Ps \geq 0.1$; Fig. 3a).

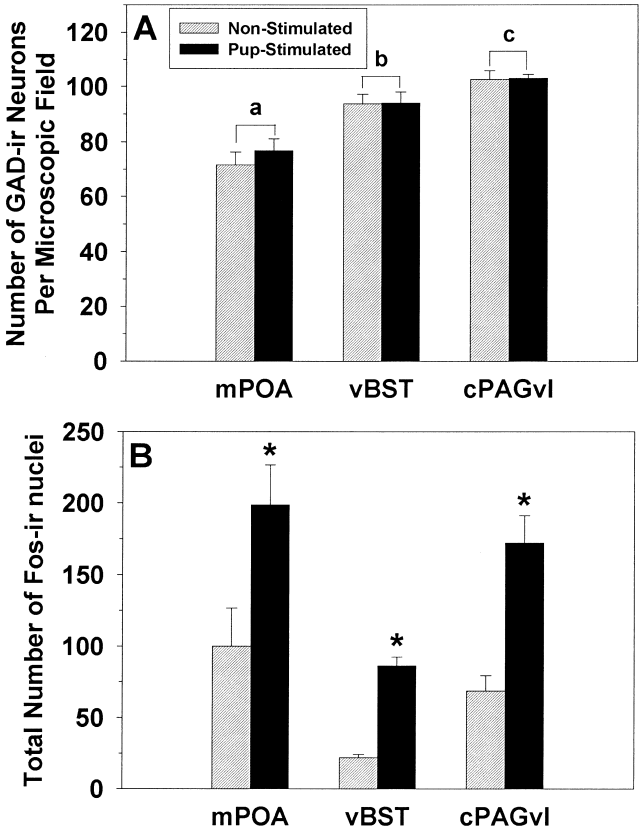

Fig. 3. Number (mean ± S.E.) of (A) GAD_{67}-IR and (B) Fos-IR cells per microscopic field in the mPOA, vBST, and $cPAG_{vl}$ of non-stimulated or pup-stimulated dams ($n = 9$). Six adjacent microscopic fields were analysed in each hemisphere for the mPOA and $cPAG_{vl}$, three fields in each hemisphere for the vBST. Significant differences between sites collapsed across group in A indicated by different letters above bracketed groups of bars ($P \leq 0.05$). In B, $^*P \leq 0.05$.

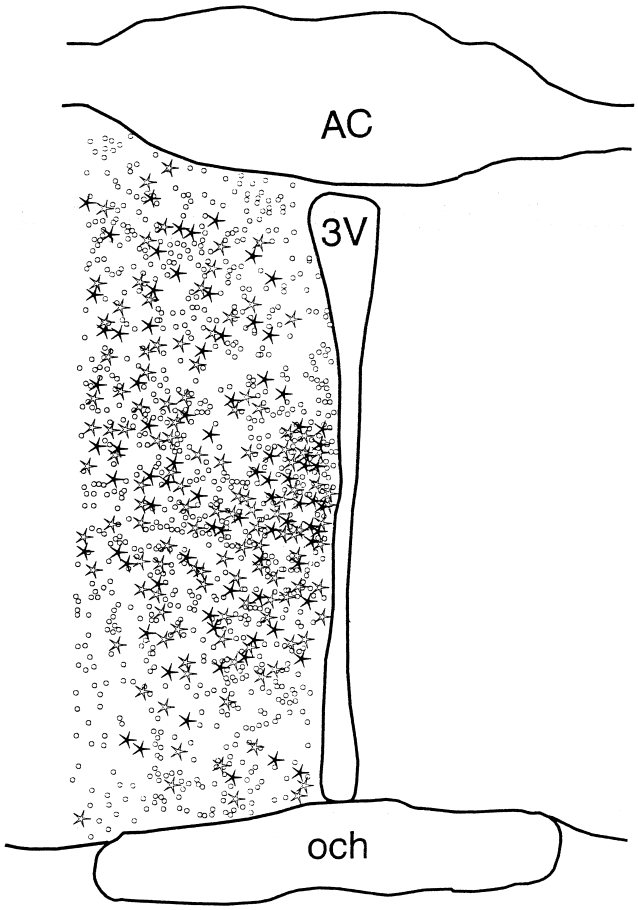

Fig. 4. Schematic reproduction of the distribution of GAD_{67}-IR (open circles), Fos-IR (open stars), and dual-labeled (black stars) neurons in the mPOA of a representative pup-stimulated dam. Each symbol represents one cell. Note that area depicted is larger than area included in data analyses. AC, anterior commisure; och, optic chiasm; 3V, third ventricle.

In the vBST, more GAD_{67}-IR neurons were found in the medial and middle thirds of the area analysed compared with the lateral third ($F_{2,\ 48} = 9.23$, $P \leq 0.0004$; Table 1, Fig. 5). There was no significant group effect for the number of GAD_{67}-IR neurons in the vBST ($P \geq 0.1$).

In the $cPAG_{vl}$, more GAD_{67}-IR neurons were located ventrally than dorsally ($F_{1,96} = 9.02$, $P \leq 0.005$) and more were located in the medial and the middle areas analysed than laterally ($F_{2,96} = 10.87$, $P \leq 0.0001$; Table 1, Fig. 6). There was also a significant dorsoventral by mediolateral effect such that the most GAD_{67}-IR neurons were found in the ventromedial field of analysis ($F_{2,96} = 14.13$, $P \leq 0.0001$). There were no significant group effects or interactions effects including group as a variable for the number of GAD_{67}-IR neurons in the $cPAG_{vl}$ ($Ps \geq 0.1$).

Fos-immunoreactive labeling

In all three sites, pup-stimulated dams had significantly more Fos-IR nuclei than non-stimulated dams. In the mPOA, pup-stimulated dams had twice as many Fos-IR cells than non-stimulated dams ($F_{1,96} = 25.345$, $P \leq 0.0001$; Fig. 3b). Collapsed across groups, more Fos-IR nuclei were found in the dorsal and medial thirds of the mPOA compared with the ventral third ($F_{2,96} = 7.31$, $P \leq 0.002$; Table 2, Fig. 4). This main effect of dorsoventral position may primarily be due to the non-stimulated dams, because there was also a significant group by dorsoventral position effect ($F_{2,96} = 3.69$, $P \leq 0.03$) in a similar pattern just for non-stimulated dams, but not for pup-stimulated dams. There were no differences in Fos-IR labeling in the mPOA according to mediolateral position ($F_{1,96} = 0.03$, $P \geq 0.6$).

In the vBST, pup-stimulated dams had four times as many Fos-IR nuclei than non-stimulated dams ($F_{1,48} = 35.75$, $P \leq 0.0001$; Fig. 3b). A significant mediolateral position effect was found such that the medial third of the vBST had more Fos-IR nuclei than the two more lateral areas of the vBST ($F_{2,48} = 5.31$, $P \leq 0.009$; Table 2, Fig. 5). There was no significant group by position effect ($F_{2,48} = 0.22$, $P \geq 0.05$).

In the $cPAG_{vl}$, pup-stimulated dams had two and a half times as many Fos-IR cells than non-stimulated dams ($F_{1,96} = 85.67$, $P \leq 0.0001$; Fig. 3b). More Fos-IR labeling was found ventrally than dorsally ($F_{1,96} = 7.91$, $P \leq 0.006$), but there was no significant difference mediolaterally ($F_{2,96} = 1.54$, $P \leq 0.2$; Table 2, Fig. 6). No significant group by position interaction effects were found ($Ps \geq 0.05$).

Glutamate decarboxylase$_{67}$-immunoreactive plus Fos-immunoreactive labeling

Many cells in all three sites analysed contained both

Table 1. Number (mean ± S.E.M.) of glutamate decarboxylase$_{67}$-immunoreactive neurons in subregions of the medial preoptic area, ventral bed nucleus of the stria terminalis, and caudal lateral and ventrolateral periaqueductal gray of non-stimulated ($n = 9$) or pup-stimulated ($n = 9$) lactating rats on day 7 postpartum

Region	Non-stimulated	Pup-stimulated
mPOA		
Dorsal*	346 ± 22	364 ± 22
Middle†	265 ± 18	302 ± 20
Ventral‡	247 ± 25	254 ± 25
Medial*	443 ± 37	495 ± 35
Lateral†	416 ± 24	426 ± 21
vBST		
Medial*	205 ± 9	194 ± 14
Middle*	208 ± 6	215 ± 16
Lateral†	167 ± 4	167 ± 9
cPAG$_{vl}$		
Dorsal*	571 ± 27	597 ± 14
Ventral†	637 ± 29	639 ± 12
Medial*	411 ± 13	456 ± 20
Middle*	426 ± 17	416 ± 14
Lateral†	372 ± 19	365 ± 14

Where there are significant main effects of position, post hoc differences between positions within each site indicated by different symbols, $P \leq 0.05$. See text for additional statistical results.

Table 2. Number (mean ± S.E.M.) of Fos-immunoreactive neurons within subregions of the medial preoptic area, ventral bed nucleus of the stria terminalis, and caudal lateral and ventrolateral periaqueductal gray of non-stimulated ($n = 9$) or pup-stimulated ($n = 9$) lactating rats on day 7 postpartum

Region	Non-stimulated	Pup-stimulated
mPOA		
Dorsal*	62 ± 18	68 ± 5
Middle*	28 ± 8	76 ± 12
Ventral†	10 ± 3	55 ± 16
Medial	50 ± 11	96 ± 15
Lateral	50 ± 16	103 ± 12
vBST		
Medial*	19 ± 3	35 ± 3
Middle†	13 ± 3	25 ± 3
Lateral†	12 ± 3	25 ± 2
cPAG$_{vl}$		
Dorsal*	32 ± 5	73 ± 10
Ventral†	37 ± 7	99 ± 10
Medial	24 ± 4	55 ± 9
Middle	26 ± 4	63 ± 7
Lateral	19 ± 4	54 ± 5

Where there are significant main effects of position, significant post hoc differences between positions within each site indicated by different symbols, $P \leq 0.05$. See text for additional statistical results.

GAD$_{67}$-IR and Fos-IR labeling. In the mPOA, over twice as many double-labeled cells were found for pup-stimulated dams than non-stimulated dams ($F_{1,96} = 56.83$, $P \leq 0.0001$; Fig. 7a). Slightly more than half of all Fos-IR cells in the mPOA were also GAD$_{67}$-IR for both groups of dams ($F_{1,89} = 0.75$, $P \geq 0.3$; Fig. 7b). The position of dual-labeled cells within the mPOA was very similar to that of the population of cells that were just Fos-IR. More dual-labeled cells were found in the dorsal two-thirds compared with the ventral third ($F_{2,96} = 11.11$, $P \leq 0.0001$) and there was no difference according to mediolateral position ($F_{1,96} = 0.10$, $P \geq 0.8$; Table 3, Fig. 4). Similar to the population of Fos-IR cells, a significant group by dorsoventral position effect was found such that only non-stimulated dams had

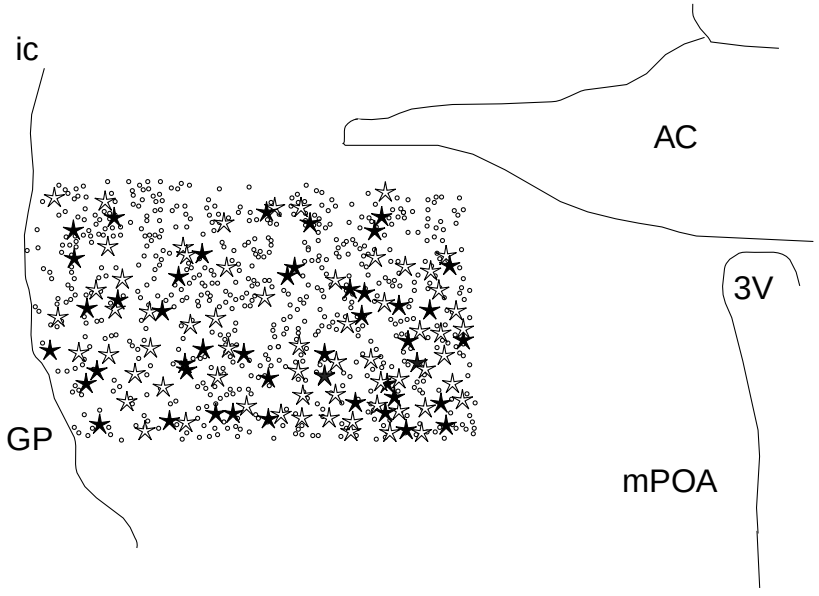

Fig. 5. Schematic reproduction of the distribution of GAD$_{67}$-IR (open circles), Fos-IR (open stars), and dual-labeled (black stars) neurons in the vBST of a representative pup-stimulated dam. Each symbol represents one cell. Note that area depicted is larger than area included in data analyses. GP, globus palladus; ic, internal capsule; AC, anterior commisure; mPOA, medial preoptic area; 3V, third ventricle.

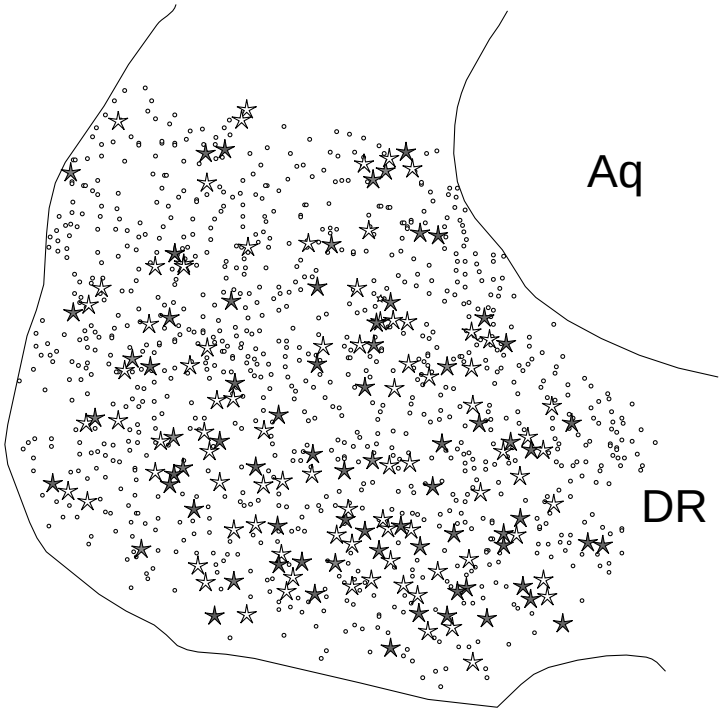

Fig. 6. Schematic reproduction of the distribution of GAD_{67}-IR (open circles), Fos-IR (open stars), and dual-labeled (black stars) neurons in the $cPAG_{vl}$ of a representative pup-stimulated dam. Each symbol represents one cell. Note that area depicted is larger than the area included in data analyses. DR, dorsal raphe; Aq, cerebral aqueduct.

more dual-labeled cells dorsally than ventrally ($F_{2,96} = 3.93$, $P \leq 0.03$).

In the vBST, Pup-Stimulated dams had almost threefold more double-labeled cells than non-stimulated dams ($F_{1,48} = 6.22$, $P \leq 0.02$; Fig. 7a). The proportion of the total Fos-IR population of neurons that were also GAD_{67}-IR was lower in pup stimulation dams (59%) than no stimulation dams (84%) ($F_{1,96} = 27.03$, $P \leq 0.0001$; Fig. 7b). Unlike the population of neurons that were labeled singly with Fos-immunoreactivity, there was no significant effect of mediolateral position on the number of double-labeled neurons for either group ($F_{2,48} = 1.71$, $P \geq 0.1$; Table 3, Fig. 5). There was also no significant group by mediolateral position effect ($F_{2,48} = 0.35$, $P \geq 0.7$).

In the $cPAG_{vl}$, pup-stimulated dams had twice as many double-labeled cells than non-stimulated dams ($F_{1,96} = 48.12$, $P \leq 0.0001$; Fig. 7a). The proportion of Fos-IR cells that were also GAD_{67}-IR was lower in pup-stimulated dams (61%) than in non-stimulated dams (73%) ($F_{1,96} = 8.85$, $P \leq 0.005$; Fig. 7b). Similar to the population of cells that were only Fos-IR, there was a significant dorsoventral position effect such that more double-labeled cells were found ventrally than dorsally ($F_{1,96} = 6.19$, $P \leq 0.02$; Table 3, Fig. 6). There was no significant mediolateral position effect ($F_{2,96} = 2.76$, $P \geq 0.05$) and no significant interaction effects ($Ps \geq 0.1$).

Correlations between behavioural and immunocytochemical data

In the mPOA of pup-stimulated dams, there were significant negative correlations between the number of double-labeled cells and the duration that dams spent licking the pups ($r^2 = -0.46$, $P \leq 0.05$) and in total activity ($r^2 = -0.47$, $P \leq 0.05$). In the vBST, a significant negative correlation was found between the number of double-labeled cells and the total time that dams spent in contact with pups ($r^2 = -0.62$, $P \leq 0.02$). No significant correlations between the behavioural and immunocytochemical data were found within the $cPAG_{vl}$.

DISCUSSION

Although several neurochemicals have been implicated in the control of particular maternal behaviours in rats,[7] the neurochemical pathways necessary for these behaviours have not been completely identified. The present results provide evidence that GABAergic neurons in the mPOA, vBST, and $cPAG_{vl}$ are an important part of these pathways. Approximately half of all cells in the mPOA, vBST, and $cPAG_{vl}$ that show c-*fos* activity after the display of maternal behaviour in lactating rats produce GAD_{67}, the enzyme that synthesizes the inhibitory neurotransmitter GABA. These three sites are necessary for the performance of particular maternal behaviours and the populations of neurons in these areas that show elevated Fos immunoreactivity after lactating rats interact with pups may be especially important for the dam's behaviour. Considering that many of these Fos-IR cells also express GAD_{67}, inhibitory mechanisms may have a prominent role in the control of maternal behaviour in female rats.

Distribution of glutamate decarboxylase$_{67}$-immunoreactive labeling

The presence of dense concentrations of GAD_{67}-IR neurons in the mPOA, vBST, and $cPAG_{vl}$ of lactating rats is consistent with many previous studies of male[5,6,26,32,49,69,79,82,92,104,113] and non-lactating female[26,28,38,39] rats. Our finding that the density

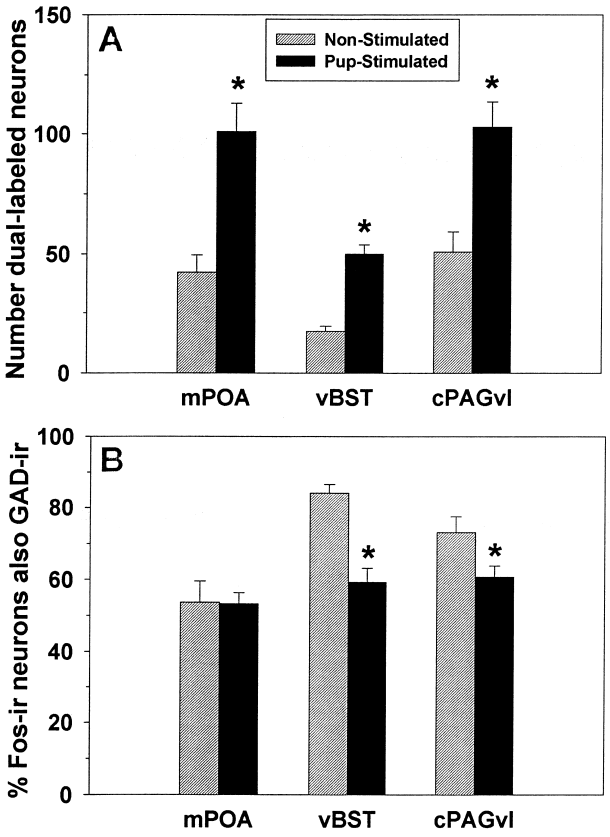

Fig. 7. (A) Number of (mean ± S.E.) of neurons dual-labeled for GAD_{67}-IR and Fos-IR in the mPOA, vBST, and $cPAG_{vl}$ of non-stimulated or pup-stimulated dams ($n = 9$). Six adjacent square microscopic areas were analysed within each hemisphere of the mPOA and $cPAG_{vl}$, three adjacent squares in each hemisphere of the vBST. (B) Percentage (mean ± S.E.) of Fos-IR neurons in each site that are also $6GAD_{67}$-IR. *$P \leq 0.05$.

of GAD_{67}-IR neurons was slightly, but significantly, different in the sites analysed may simply be the result of a different number of neurons, regardless of phenotype, in these areas.

Distribution of Fos-immunoreactive labeling

The presence of high levels of c-*fos* activity in the mPOA and vBST after the display of maternal behaviour in lactating rats is consistent with previous reports, as was the magnitude of the increase in Fos-IR nuclei in these dams.[24,57,72,73,75,77,112] Furthermore, our results are generally consistent with schematic representations of the location of Fos-IR nuclei in the mPOA and vBST of maternal lactating rats from some previous reports.[72,73,77]

Pup-stimulated dams also showed significantly more Fos-IR nuclei in the $cPAG_{vl}$ compared with non-stimulated dams, consistent with previous reports.[52,53] The greater amount of Fos-IR labeling within the ventral part of the $cPAG_{vl}$ may suggest that this ventral subregion within the greater population of $cPAG_{vl}$ neurons may be particularly responsive to sensory cues from pups. However, the relative increase in c-*fos* activity in the $cPAG_{vl}$ after maternal behaviour found in the present study (~2.5 fold) was much smaller than that found previously by Lonstein and Stern[52,53] (~6–9 fold) but was quite similar to that found by Li *et al.*[50] (~3.5 fold). One possible explanation for these differences is that whereas Lonstein and Stern[52,53] examined maternal behaviour in Long–Evans rats, albino rats were used in the present study and in the report by Li *et al.*[50] Rat

Table 3. Number (mean ± S.E.M.) and distribution of neurons that were both Fos-immunoreactive and glutamate decarboxylase in the medial preoptic area, ventral bed nucleus of the stria terminalis, and caudal lateral and ventrolateral periaquedeuctal gray of non-stimulated ($n = 9$) or pup-stimulated ($n = 9$) lactating rats on day 7 postpartum

Region	Non- stimulated	Pup-stimulated
mPOA		
Dorsal*	26 ± 5	36 ± 3
Middle*	11 ± 3	38 ± 6
Ventral†	5 ± 1	27 ± 5
Medial	21 ± 5	49 ± 7
Lateral	22 ± 5	53 ± 5
vBST		
Medial	12 ± 2	20 ± 2
Middle	13 ± 3	27 ± 11
Lateral	7 ± 1	14 ± 1
$cPAG_{vl}$		
Dorsal*	24 ± 4	42 ± 6
Ventral†	28 ± 5	55 ± 4
Medial	19 ± 4	33 ± 5
Middle	19 ± 3	35 ± 4
Lateral	13 ± 3	29 ± 2

Where significant main effects for position exist, significant post hoc differences between positions within each site indicated by different symbols, $P \leq 0.05$. See text for additional statistical results.

strains differ in their sensory perception,[17] neural characteristics,[25,93] and maternal behaviour.[64,66] These differences may extend to neural responses to sensory cues received from pups, including suckling. Indeed, Long–Evans and albino dams show different nursing responses to suckling pups and there is circumstantial evidence that Long–Evans and albino pups differ in the strength or patterning of their suckling.[66] Since the $cPAG_{vl}$ acts as a sensorimotor integration site for the kyphotic nursing posture,[52,53] these factors could conceivably contribute to strain differences in c-*fos* activity within this area of the lactating rat brain.

Co-expression of neural Fos and glutamate decarboxylase$_{67}$ in maternal lactating rats

The distribution of neurons in the mPOA and $cPAG_{vl}$ of maternally-acting dams that co-expressed the GAD_{67} and Fos proteins was quite similar to the distribution of neurons that expressed only the Fos protein. In contrast, within the vBST slightly more neurons containing only Fos immunoreactivity were situated medially whereas dual-labeled neurons were found throughout this structure. In pup-stimulated dams, more than half (53–61%) of all Fos-IR cells in the three sites analysed were also GAD_{67}-IR. Although it is impossible to determine the exact number of dual-labeled neurons in the brains of maternally-acting dams that were specifically stimulated by interactions with pups, a comparison between the increase in double-labeled neurons relative to the increase in Fos-IR cells in pup-stimulated dams over non-stimulated dams indicates that approximately half or more of the increase in the number of Fos-IR neurons in pup-stimulated dams occurred in neurons that also expressed GAD_{67} (60% in mPOA, 48% in the vBST, 50% in $cPAG_{vl}$). Physical

interaction with pups and the display of maternal behaviour, therefore, results in Fos expression in many GABAergic neurons.

Populations of neurons that express Fos after the receipt of peripheral sensory stimulation or the performance of a particular behaviour may be especially important for these processes. This is bolstered by the ability of central infusion of c-*fos* antisense oligonucleotides to alter the display of numerous behaviours,[36,37,40,41,68] including maternal behaviour in sheep.[14] In most cases, however, it is unknown how behaviour may be influenced by Fos-expressing neurons. In maternally-behaving rats, the presence of GAD_{67} in neuronal somata that also contain Fos immunoreactivity indicates that many neurons that are activated during interactions with pups release GABA and produce inhibitory postsynaptic potentials. Despite the fact that GABAergic neurons may co-express other neurochemicals that may potentially modulate or temper their inhibitory output from GABAergic neurons,[3,4,10,27,33,45,48,67,78,80,89] our findings suggest that many Fos-IR neurons in the mPOA, vBST and $cPAG_{vl}$ of maternal dams may be inhibitory in nature. Such neurons could provide a permissive effect on the dam's behaviour by inhibiting neurons that, themselves, inhibit maternal behaviour (disinhibition). Disinhibition may be a mechanism by which non-parental animals are induced to become parental and numerous motor activities, including other reproductive functions in both female[62] and male[87] rats, are mediated at least in part via disinhibition.[11] There are a myriad of inhibitory influences on parental behaviour in rats that may require inactivation, and disinhibition necessary for parental behaviour could act at many levels of the CNS. Since GABAergic neurons can be either short inhibitory interneurons or longer projection neurons,[20,65,83,88,104,107] GABAergic influences from the mPOA, vBST, or $cPAG_{vl}$ may be found both in close proximity to these areas as well as much further away. Our finding that more double-labeled cells were found in the dorsal region of the mPOA and medial areas of the vBST suggests that inhibitory output from these subregions may be especially important sources of GABAergic output. Relatively small lesions that destroy the dorsal mPOA are particularly likely to impair maternal behaviours in lactating rats compared with lesions elsewhere in the mPOA.[42] However, others have not found a correlation between the location of small lesions within the mPOA and impairments the maternal behaviour displayed by virgin juvenile or adult rats continuously exposed to pups.[44] Furthermore, the largest relative increase in the number of double-labeled cells for pup-stimulated dams compared with non-stimulated dams appeared in the ventral mPOA. The only study investigating the effects of vBST lesions on maternal behaviour in lactating rats did not distinguish between medial and lateral areas of this structure.[74]

Prior to disinhibition of striatal motor mechanisms necessary for the display of active maternal behaviours,[11,100] the normal aversion to olfactory and other stimuli from pups must be overcome before previously non-parental animals will act parentally.[22,23] This may occur naturally in newly parturient mothers by changes in GABAergic transmission to olfactory sites[47,70] that possibly arises from the mPOA or vBST.[29] Neural sites such as the ventromedial hypothalamus and PAG that mediate aggression, fear, or anxiety may also require inactivation before contact with the young is initiated.[8,34,55] Additionally, the alternation between active maternal behaviours mediated by the mPOA and vBST and quiescent kyphosis controlled by the $cPAG_{vl}$ may require reciprocal inhibition between these sites to prevent inappropriate behavioural responding by the dam. This is supported by the fact that electrical stimulation of the mPOA inhibits neuronal firing in the $cPAG_{vl}$ and vice-versa.[59,61] Lastly, the ventromedial nucleus of the hypothalamus (VMH) is inhibitory for maternal behaviour[8] and 10–25% of the Fos-IR neurons in the mPOA and vBST directly project to the area of the basal hypothalamus that includes the VMH.[75] GABAergic inhibition of the VMH, however, probably does not arise solely from the mPOA because deafferentiation from the mPOA and vBST does not significantly reduce GAD activity in the VMH.[106] GAD activity in the VMH is reduced after disconnection from the lateral hypothalamus,[106] though, which receives afferents from the mPOA necessary for maternal behaviour.[76] Although the present experiment focused on three neural sites that are facilitatory for maternal behaviours, it would also be interesting to examine whether some of the Fos-IR cells in neural sites that are inhibitory for maternal behaviour (e.g. medial amygdala[23]) are also GAD_{67}-IR when lactating dams interact with pups. One would expect that the numbers of such double-labeled cells would be low, particularly in projection neurons, since they could potentially interfere with maternal responding.

GABAergic neurons in the $cPAG_{vl}$ may provide inhibitory mechanisms that are necessary for the normal display of nursing behaviours. Neurons within the $cPAG_{vl}$ are tonically inhibited by GABAergic neurons from yet unknown origins (possibly the mPOA) to prevent the display of kyphosis when lactating dams are not being suckled by pups. This tonic inhibition must be overcome to allow for the dam's assumption of this nursing posture. This hypothesis is supported by the ability of infusion of the $GABA_A$ receptor antagonist bicuculline into $cPAG_{vl}$ to produce kyphosis in dams interacting with nonsuckling pups, a stimulus that normally does not elicit this posture.[85] Conversely, suckling becomes unable to elicit kyphosis after $cPAG_{vl}$ infusion of the $GABA_A$ agonist muscimol.[85] Since the present results indicate that suckling produces c-*fos* activity predominantly within GABAergic neurons of the suckling-responsive region of the $cPAG_{vl}$, it is most likely that suckling provides an excitatory input to GABAergic cPAG neurons which, when stimulated, disinhibit premotor neurons in the medulla to allow for the display of kyphosis. Some ventral PAG neurons that project to premotor areas of the medulla are in fact GABAergic[12,82] and GABAergic neurons found in premotor areas of the medulla can potentially inhibit spinal motorneurons.[43,51,90]

As noted above, GAD activity in many areas of the brain, including the mPOA and PAG, can be influenced by ovarian hormones.[26,38,39,62,84,86,111] Since dramatic changes in ovarian hormone activity occur throughout pregnancy and lactation,[7] it is possible that these hormonal fluctuations influence the onset or postpartum display of maternal behaviour by producing changes in GABAergic neurotransmission in cells that are important for these behaviours. This is supported by the fact that a substantial number of Fos-IR cells in the mPOA, vBST and $cPAG_{vl}$ of maternally behaving rats also express ERα.[58]

CONCLUSION

The presence of a large number of neurons in the mPOA, vBST, and $cPAG_{vl}$ that co-express Fos and GAD_{67} after the

display of maternal behaviour in lactating rats indicates that inhibitory mechanisms are a potentially critical part of the neural circuitry involved in the display of particular components of maternal behaviour, as well as for a multitude of other physiological processes in lactating rats. Areas of the brain important for maternal responding, or its suppression, that receive this inhibitory input remain to be determined.

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