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

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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 isof orm of glutamate decarboxylase, the synthe sizing 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. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

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. 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. In contrast, the lateral and ventrolateral regions of the midbrain periaqueductal gray (cPAGvl) are necessary for quiescent nursing behaviour in the typical kyphotic (upright crouched) posture, which requires sufficient suckling by the pups.

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 cPAGvl 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. Levels of Fos labeling in these sites are not affected by the presence or absence of suckling. Alternatively, suckling by the pups and subsequent nursing by the dam are necessary for high levels of c-fos activity in the cPAGvl of dams that interact with pups. Furthermore, the number of Fos-immunoreactive (Fos-IR) cells in the cPAGvl are positively correlated with the duration of kyphosis displayed by the dam and are...
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 cPAGvl, synthesize the inhibitory neurotransmitter GABA,2,26 it may be that many Fos-IR neurons in these sites are GABAergic. Activity of glutamate decarboxylase (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),26,30,63,84,86,111 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 activities.53

We examined this possibility by determining the extent of overlap between the populations of cells in the mPOA, vBST, and caudal ventrolateral periaqueductal gray (cPAGvl) 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 (GADGF). We immunocytochemically detected GADGF because, unlike the smaller 65,000 mol. wt isoform of GAD (GAD53) that is found primarily in nerve terminals, the larger GAD isoform can be found within the cytoplasm of neuronal somata60 and therefore can be clearly localized with the Fos protein, which is primarily found in the nucleus.13 Although GAD53 may be the isoform that is particularly important for extracellular GABA release,60 the vast majority of neurons that express GAD53 in their terminals also express GAD67 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 × 28 × 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 ~22 ± 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 and 30–50% of neurons in the cPAGvl, synthesize the inhibitory neurotransmitter GABA,2,26 it may be that many Fos-IR neurons in these sites are GABAergic. Activity of glutamate decarboxylase (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),26,30,63,84,86,111 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 activities.53

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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 exposed 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 previously33 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, tail-biting and rough-and-tumble play, self-grooming, exploration, nest-building, licking, and perching. None of these behaviours were observed to display over pups: kyphosis,33 or upright crouching over the litter in a high or low-arched posture,52 lying 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 receipt16 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% parafomaldehyde (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 (GADGF-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 incubated in 0.5% H2O2 for 15 min and 5 μM Tris 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 antisem 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 GADGF primary antisem indicated that Triton X-100 reduced GADGF immunostaining. In pilot experiments, however, we found that concentrations of Triton X-100 only greater than 0.15% reduced GADGF immunostaining whereas concentrations of Triton X-100 greater than 0.03% did not further enhance Fos immunostaining. After incubation in the Fos primary antisem, 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′-diaminobenzidine with 1.5% nickel ammonium sulfate, 0.04% ammonium chloride, 0.0004% glucose oxidase, and 0.15% -glucose oxidase in TBS, which provided a dark purple or black nuclear stain. Sections were rinsed five times in TBS and then run for GADGF immunocytochemistry with a rabbit primary polyclonal antisem that recognizes GADGF (AB108, 9).
1:4000; Chemicon, Temecula, CA, USA). The GAD67 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 GAD67-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, GAD67-IR, and Fos-IR plus GAD67-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 GAD67-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 staining restricted to the cell nucleus and GAD67-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 GAD67. 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 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 ventral medial 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 thalmo-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 Swanson105 (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 GAD67-IR intensities was small.

Fig. 1. Diagrammatic representation of the areas of the (A) mPOA and vBST, and (B) cPAG vl analysed for Fos-IR and GAD67-IR labeling. Black boxes represent the three (vBST) or six (mPOA, cPAG vl) adjacent square microscopic areas analysed. Aco, anterior commissure; 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 chiasm. SC, superior colliculus; vBST, ventral bed nucleus of the stria terminalis; IV, trochlear nucleus. Modified from Ref. 105.
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 ($P_s > 0.1$), 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 ($7.2 \pm 0.4$ pups) to the nest within $3 \pm 1$ min and spent $54 \pm 1$ min in physical contact with them. Dams actively hovered over the pups for $24 \pm 2$ min of this time while licking them ($15 \pm 2$ min) and self-grooming ($2 \pm 0$ min). All dams displayed prolonged periods of quiescent nursing behaviour (total duration $= 30 \pm 2$ min) that included $5 \pm 1$ 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 ($F_{2,96} = 4.44, P = 0.04$). 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 ($Ps > 0.1$; Fig. 3a).
In the vBST, more GAD67-IR neurons were found in the medial and middle thirds of the area analysed compared with the lateral third \((F_{2, 48} = 9.23, P < 0.0004;\) Table 1, Fig. 5). There was no significant group effect for the number of GAD67-IR neurons in the vBST \((P > 0.1)\).

In the cPAG vl, more GAD67-IR neurons were located ventrally than dorsally \((F_{1, 96} = 9.02, P < 0.005)\) and more were located in the medial and the middle areas analysed than laterally \((F_{2, 96} = 10.87, P < 0.0001;\) Table 1, Fig. 6). There was also a significant dorsoventral by mediolateral effect such that the most GAD67-IR neurons were found in the ventromedial field of analysis \((F_{2, 96} = 14.13, P < 0.0001).\) There were no significant group effects or interactions including group as a variable for the number of GAD67-IR neurons in the cPAG vl \((P > 0.1).\)

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 \((F_{1, 96} = 25.345, P = 0.0001;\) 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 \((F_{2, 96} = 7.31, P = 0.002;\) 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 \((F_{2, 96} = 3.69, P = 0.03)\) 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 \((F_{1, 96} = 0.03, P > 0.05).\)

In the vBST, pup-stimulated dams had four times as many Fos-IR nuclei than non-stimulated dams \((F_{1, 48} = 35.75, P = 0.0001;\) 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 \((F_{2, 48} = 5.31, P = 0.009;\) Table 2, Fig. 5). There was no significant group by position effect \((F_{2, 48} = 0.22, P > 0.05).\)

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

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 decarboxylase67-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

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<th>Region</th>
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<td>Lateral†</td>
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<tr>
<td>Lateral†</td>
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<td>365 ± 14</td>
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Where there are significant main effects of position, post hoc differences between positions within each site indicated by different symbols, P ≤ 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

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<td>25 ± 2</td>
</tr>
<tr>
<td>cPAGvl</td>
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<td></td>
</tr>
<tr>
<td>Dorsal*</td>
<td>32 ± 5</td>
<td>73 ± 10</td>
</tr>
<tr>
<td>Ventral†</td>
<td>37 ± 7</td>
<td>99 ± 10</td>
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<td>Medial</td>
<td>24 ± 4</td>
<td>55 ± 9</td>
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<tr>
<td>Middle</td>
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<td>63 ± 7</td>
</tr>
<tr>
<td>Lateral</td>
<td>19 ± 4</td>
<td>54 ± 5</td>
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Where there are significant main effects of position, significant post hoc differences between positions within each site indicated by different symbols, P ≤ 0.05. See text for additional statistical results.

GAD67-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_{1,106} = 56.83, P = 0.0001; Fig. 7a). Slightly more than half of all Fos-IR cells in the mPOA were also GAD67-IR for both groups of dams (F_{1,90} = 0.75, P = 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_{2,106} = 11.11, P = 0.0001) and there was no difference according to mediolateral position (F_{1,90} = 0.10, P ≥ 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 GAD67-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 pallidus; ic, internal capsule; AC, anterior commissure; mPOA, medial preoptic area; 3V, third ventricle.](image-url)
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 \((F_{1,48} = 6.22, P \leq 0.02; \text{Fig. 7a})\). 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\%) \((F_{1,96} = 27.03, P \leq 0.0001; \text{Fig. 7b})\). 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 \((F_{2,48} = 1.71, P \geq 0.1; \text{Table 3, Fig. 5})\). 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 \((F_{1,96} = 48.12, P \leq 0.0001; \text{Fig. 7a})\). 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\%) \((F_{1,96} = 8.85, P \leq 0.005; \text{Fig. 7b})\). 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 \((F_{1,96} = 6.19, P \leq 0.02; \text{Table 3, Fig. 6})\). There was no significant mediolateral position effect \((F_{2,96} = 2.76, P \geq 0.05)\) and no significant interaction effects \((P \geq 0.1)\).

**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
of GAD<sub>67</sub>-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. Furthermore, our results are generally consistent with schematic representations of the location of Fos-IR nuclei in the mPOA and cPAG<sub>vl</sub> in maternal lactating rats from some previous reports. Pup-stimulated dams also showed significantly more Fos-IR nuclei in the cPAG<sub>vl</sub> compared with non-stimulated dams and the distribution of Fos-IR in the mPOA, vBST, and cPAG<sub>vl</sub> of non-stimulated or pup-stimulated dams indicates that approximately half or more of the increase in double-labeled neurons relative to the increase in Fos-IR cells in pup-stimulated dams over non-stimulated pups differ in the strength or patterning of their 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. Since the cPAG<sub>vl</sub> acts as a sensorimotor integration site for the kypthetic nursing posture, 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<sub>67</sub> in maternal lactating rats**

The distribution of neurons in the mPOA and cPAG<sub>vl</sub> of maternally-acting dams that co-expressed the GAD<sub>67</sub> 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<sub>67</sub>-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 puppet-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<sub>67</sub> (60% in mPOA, 48% in the vBST, 50% in cPAG<sub>vl</sub>). Physical

<table>
<thead>
<tr>
<th>Region</th>
<th>Non-stimulated</th>
<th>Pup-stimulated</th>
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<tr>
<td>mPOA</td>
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<tr>
<td>Dorsal*</td>
<td>26 ± 5</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>Middle*</td>
<td>11 ± 3</td>
<td>38 ± 6</td>
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<tr>
<td>Ventral†</td>
<td>5 ± 1</td>
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<tr>
<td>Medial</td>
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<td>53 ± 5</td>
</tr>
<tr>
<td>vBST</td>
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<tr>
<td>Medial</td>
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</table>

Where significant main effects for position exist, significant post hoc differences between positions within each site indicated by different symbols, P ≤ 0.05. See text for additional statistical results.
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, including maternal behaviour in sheep. In most cases, however, it is unknown how behaviour may be influenced by Fos-expressing neurons. In maternally-behaving rats, the presence of GAD67 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 neurotransmitters that may potentially modulate or temper their inhibitory output from GABAergic neurons, 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, are mediated at least in part via disinhibition. 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, 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. 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. 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.

Prior to disinhibition of striatal motor mechanisms necessary for the display of active maternal behaviours, the normal aversion to olfactory and other stimuli from pups must be overcome before previously non-parental animals will act parentally. This may occur naturally in newly parturient mothers by changes in GABAergic transmission to olfactory sites that possibly arises from the mPOA or vBST. 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. 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. Lastly, the ventromedial nucleus of the hypothalamus (VMH) is inhibitory for maternal behaviour 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. GABAergic inhibition of the VMH, however, probably does not arise solely from the mPOA because deafferentation from the mPOA and vBST does not significantly reduce GAD activity in the VMH. GAD activity in the VMH is reduced after disconnection from the lateral hypothalamus, though, which receives afferents from the mPOA necessary for maternal behaviour. 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) are also GAD2-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 the 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. Conversely, suckling becomes unable to elicit kyphosis after cPAG vl infusion of the GABA A agonist muscimol. Since the present results indicate that suckling produces c-fos activity predominantly within GABAergic neurons of the sucking-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 and GABAergic neurons found in premotor areas of the medulla can potentially inhibit spinal motorneurons.

As noted above, GAD activity in many areas of the brain, including the mPOA and PAG, can be influenced by ovarian hormones. Since dramatic changes in ovarian hormone activity occur throughout pregnancy and lactation, 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 ERa.

**CONCLUSION**

The presence of a large number of neurons in the mPOA, vBST, and cPAG vl that co-express Fos and GAD at 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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REFERENCES


