Amylin is a novel neuropeptide with potential maternal functions in the rat

Éva Rebeka Szabó, Melinda Cservenák, and Arpád Dobolyi[1]

Neuromorphological and Neuroendocrine Research Laboratory, Department of Anatomy, Histology, and Embryology, Semmelweis University and the Hungarian Academy of Sciences, Budapest, Hungary

ABSTRACT **Amylin, a 37-aa pancreatic peptide, was found to be expressed in the preoptic area of mother rats in our recent microarray study. Here, we report a marked increase in amylin expression around parturition and show that amylin mRNA level remains elevated as long as the pups are not removed from the dams. Amylin expression is also induced in maternally behaving (sensitized) nonlactating but not in nonsensitized nulliparous females or in females that did not become maternal despite the sensitization procedure. Immunohistochemistry verified the increased amylin peptide expression in maternally behaving rats and demonstrated the same expression pattern of amylin as *in situ* hybridization histochemistry. Ovariectomy had no effect on the activation of amylin neurons, suggesting sexual steroid-independent mechanisms. In subsequent functional experiments, mothers were separated from their pups for 22 h. On return of the pups, neuronal activation was found in the mother's preoptic area, with a distribution pattern similar to amylin-expressing neurons. Subsequent double labeling revealed that 86–93% of amylin neurons were activated by pup exposure. The results implicate amylin in the control of maternal adaptations, possibly exerting its actions on maternal behaviors *via* amylin receptors present in brain regions to which preoptic neurons project.—Szabó, E. R., Cservenák, M., Dobolyi, A. Amylin is a novel neuropeptide with potential maternal functions in the rat. *FASEB J.* 26, 272–281 (2012). www.fasebj.org**

Key Words: preoptic area · hypothalamus · postpartum mother · peptide expression

Amylin is a 37-aa peptide that belongs to the calcitonin peptide family. It has roughly 50% sequence homology to calcitonin gene-related peptide, and 20–30% homology to other members of the calcitonin family of peptides, including calcitonin, adrenomedullin, and intermedin/adrenomedullin 2 (1). Amylin is encoded in one copy by a single gene (Unigene code for rat: Rn.11394), which does not encode any other known peptide (2). Amylin has 3 receptors, each of which consists of a "family B " G-protein-coupled receptor, the calcitonin receptor that is coupled to one of 3 single-transmembrane domain receptor activity-modifying proteins (3, 4). Amylin expression is most abundant in the pancreas. Amylin has been shown to be released with insulin from pancreatic β cells (5). The most established function of serum amylin is the inhibition of food intake as a satiating hormone (6, 7). In addition, amylin expressed in the dorsal root ganglia (8) may play a role in the transmission of nociceptive information (9). A recent microarray study identified a robust increase in the level of amylin mRNA in the preoptic area of mother rats (10). The preoptic area plays a central role in the control of maternal adaptations (11), based primarily on the complete absence of all maternal behaviors following preoptic lesions (12, 13) and the extensive c-Fos activation in neurons of the preoptic area in postpartum dams (14–16). Therefore, we hypothesized that amylin is a neuropeptide and with specific maternal functions. To test this hypothesis, we first investigated the expression pattern of amylin during late pregnancy and throughout lactation, using *in situ* hybridization and immunohistochemistry. For comparison, amylin immunoreactivity was also assessed in the maternal pancreatic islets. Subsequently, the induction of amylin was investigated in maternally behaving sensitized virgin females, using *in situ* hybridization, quantitative RT-PCR, and immunolabeling. These maternally sensitized rats do not lactate and, thus, provide a model to separate metabolic regulation from regulation of maternal behaviors (17, 18). Estradiol and progesterone levels rise during pregnancy, but decreased levels of these hormones are detected during lactation (19). Therefore, the effect of ovariectomy on amylin levels, as well as on sensitization-elicited induction of amylin, was investigated. Finally, the participation of amylin neurons was examined in maternal control. Pups were returned to mothers after a day of separation, which evokes *c-fos* expression in the preoptic area of rat dams (14–16). The distribution patterns of *c-fos*- and amylin-expressing neurons, as well as the *c-fos* activation in amylin neurons, were investigated

[1] Correspondence: Laboratory of Neuromorphology, Department of Anatomy, Histology and Embryology, Semmelweis University, Tüzolto u. 58, Budapest, H-1094, Hungary. E-mail: dobolyi@ana.sote.hu

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using double fluorescent immunocytochemistry and a combination of immunocytochemistry and *in situ* hybridization histochemistry.

MATERIALS AND METHODS

Animals

All animal experimentation was conducted in accordance with the U.S. National Research Council Guide for the Care and Use of Laboratory Animals. Experiments were carried out according to protocols approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at Semmelweis University and met the guidelines of the Animal Hygiene and Food Control Department, Ministry of Agriculture, Hungary. A total of 79 adult female Wistar rats (260–340 g body weight; Charles River Laboratories, Budapest, Hungary) were used. All of the animals were 90–120 d old when sacrificed. Animals were kept in standard laboratory conditions with a 12-h light-dark cycle. Pregnant and postpartum mothers, maternally sensitized rats, and their experimental control counterparts were individually housed. Three mother rats were excluded from the study because they delivered <6 pups or because some of their pups died. The number of pups was adjusted to 10 within 2 d of delivery. Rats were anesthetized with an intramuscular injection of an anesthetic mix containing 66 mg/kg ketamine and 13 mg/kg xylazine for surgery, perfusions, and dissections.

Ovariectomy

Anesthetized nulliparous female rats (n=16) were ovariectomized through bilateral upper flank incisions (20). The ovarian bundles were tied off, and the ovaries were removed. The fascia was closed with sutures, and the skin was closed with metal clips. The animals were allowed to recover for 2 wk before the initiation of maternal sensitization.

Maternal sensitization

Adult virgin females were stimulated to express maternal behavior by continuous exposure to young pups. During this sensitization period, the rats were exposed to 3 pups for 24 h each day. The freshly fed pups (3–14 d of age) were scattered in the corner of the home cage opposite the females' sleeping area. One rat cannibalized pups and was excluded from the study. The pups were removed and replaced with freshly fed pups daily, as described previously (17, 18). Females were considered maternal when they had retrieved pups within 5 min during tests on 2 consecutive days and had exhibited crouching over them and licking. Fourteen of 16 rats reached this criterion within 4 to 8 d.

Pup exposure of mother rats for the *c-fos* activation study

Rat dams (n=16) were deprived of pups on d 8 and 9 postpartum at 11:00 AM. The following day at 9:00 AM, the pups were returned to the cages of 8 of the mother rats, whereas 8 control rats remained isolated. All 8 mothers accepted the pups, and suckling started within 10 min. All animals were sacrificed 24 h after removing the pups (*i.e.*, 2 h after returning the pups to the 8 mothers for suckling). Animals were perfused transcardially and processed for c-Fos and amylin double immunocytochemistry and c-Fos immunocytochemistry combined with *in situ* hybridization histochemistry for amylin.

Microdissection of brain tissue samples

Brains of 8 maternally sensitized nulliparous females and 8 age-matched nonsensitized control female rats were removed. Thick coronal brain sections that included the preoptic area were prepared with razor blade cuts immediately rostral to the optic chiasm and 2 mm caudal to this level. A horizontal cut immediately above the anterior commissure and sagittal cuts 2 mm lateral to the midline on both sides of the brain were used to dissect tissue blocks that contained the preoptic area of the hypothalamus, as well as small parts of adjacent brain structures, including parts of the diagonal band of Broca, the anterior commissure, the optic chiasm, and the ventral pallidum. In this work, however, we refer to this tissue block as the preoptic area. The dissected tissue samples were quickly frozen on dry ice, and stored at −80°C.

Real-time RT-PCR

Total RNA was isolated from the microdissected preoptic area using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After diluting RNA to 2 μg/μl, it was treated with amplification grade DNase I (Invitrogen), and cDNA was synthesized with a Superscript II reverse transcriptase kit (Invitrogen), according to the manufacturer's instructions. After 10-fold dilution, 2.5 μl of the resulting cDNA was used as a template in PCR reactions using SYBR Green dye (Sigma, St. Louis, MO, USA). The PCR reactions were performed with iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA, USA) in total volumes of 12.5 μl under the following conditions: 95°C for 3 min, followed by 35 cycles of 95°C for 0.5 min, 60°C for 0.5 min and 72°C for 1 min. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the housekeeping gene. The primers for amylin (ACATGTGCCACACAACGTCT and ACAAACACAGCAAGCACAGG, corresponding to 222–241 and 493–512 bp of GenBank accession number NM_012586) and GAPDH (TGCCACTCAGAAGACTGTGG and gTCCTCAGTGTAGCCCAGGA, corresponding to 540–559 and 812–831 bp of GenBank M17701) were used at 300 nM final concentration. Cycle threshold (C_T) values were obtained from the linear region of baseline-adjusted amplification curves. Standard curves, obtained by measuring dilution series, were used to calculate the amount of cDNA in the samples. Statistical comparisons (Prism 5 for Windows, GraphPad, La Jolla, CA, USA) were made using Student's t test.

In situ hybridization histochemistry for amylin

Preparation of the *in situ* hybridization probe was performed as described previously (21). Briefly, the PCR product of amylin from the RT-PCR experiment was purified from gel, inserted into TOPO TA cloning vectors (Invitrogen), and transformed chemically into competent bacteria. Selected plasmids were applied as templates in PCR reactions, using the primer pair specific for amylin, with the reverse primers also containing a T7 RNA polymerase recognition site. Finally, the identity of the cDNA probe was verified by sequencing.

Brains of 3 rats in each of the following groups were removed, and the fresh tissue was quickly frozen on dry ice: d 21 of pregnancy; d 1, 9, and 23 after parturition; ovariectomized; ovariectomized sensitized; and age-matched nulliparous control female rats. Experiments were performed as

described previously (22). Briefly, serial coronal sections (12 μm) were cut using a cryostat +3.0 to −3 mm from the bregma level, mounted on positively charged slides (SuperfrostPlus, Fisher Scientific, Pittsburgh, PA, USA), dried, and stored at −80°C until use. *In situ* hybridization protocols are described in detail online (http://intramural.nimh.nih.gov/lcmr/snge/Protocols/ISHH/ISHH.html). Antisense [^{35}S]UTP-labeled riboprobes were generated using T7 RNA polymerase of the MAXIscript transcription kit (Ambion, Austin, TX, USA). These probes were used for hybridization at 10^6 dpm/slide. Every 18th coronal section was hybridized to allow the visualization of amylin mRNA-expressing neurons at 216-μm distances. Following hybridization and washes, slides were dipped in NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY, USA) and stored at 4°C for 3 wk for autoradiography. Then, the slides were developed and fixed with Kodak Dektol developer and Kodak fixer, respectively, counterstained with Giemsa, and coverslipped with Cytoseal 60 (Stephens Scientific, Riverdale, NJ, USA).

Quantitation of *in situ* hybridization data

Amylin mRNA-expressing neurons, above which >9 autoradiography grains (3 times the average background) were detected, were typically present in 3 coronal sections cut at 216-μm distances. The total number of these cells was counted on d 21 of pregnancy and 1, 9, and 23 d after parturition. In addition, the number of autoradiography grains was counted in 20–20 evenly distributed but otherwise randomly selected amylin mRNA-expressing neurons in each of the 3–3 brains on d 21 of pregnancy and 1, 9, and 23 d after parturition. Both the total number of amylin mRNA-expressing neurons in the 3 consecutive sections and the average number of autoradiography grains per cell were calculated on one side of the 3–3 brain sections. Statistical analyses were performed using Prism 5 for Windows (GraphPad). Both cell numbers and the number of autoradiography grains in the 4 groups (d 21of pregnancy and 1, 9, and 23 d after parturition) were compared using 1-way ANOVA followed by Tukey's multiple comparison *post hoc* test.

Tissue collection for immunolabeling

Rats (n=31) were deeply anesthetized and perfused transcardially with 150 ml saline followed by 300 ml 4% paraformaldehyde prepared in phosphate buffer (PB; pH 7.4). Brains, and, in some cases, pancreases, were removed and postfixed in 4% paraformaldehyde for 24 h and then transferred to PB containing 20% sucrose for 2 d. Serial coronal brain sections were cut at 50 μm on a sliding microtome between 3.0- and −3.0-mm bregma levels, while serial sections from pancreas were cut at 20 μm on a cryostat.

Amylin immunocytochemistry

Every fourth 50-μm-thick free-floating brain section of 3 primiparous lactating mothers and 3 primigravid mothers on d 21 of pregnancy, 3 sensitized, 2 pup-exposed nonsensitized, and 3 control nulliparous females, as well as pancreas sections from 2 lactating mothers and 2 mothers deprived, were immunostained using an anti-amylin antiserum [rabbit anti-amylin (rat), catalog no. T-4146.0050; Bachem, Bubendorf, Switzerland]. Sections were pretreated in PB containing 0.5% Triton X-100 and 3% BSA for 1 h. Sections were then incubated in anti-amylin antiserum (1:5000) at room temperature for 2 d. Following the application of the primary antibody, sections were incubated in biotin-conjugated goat anti-rabbit secondary antibody (1:1000; Vector Laboratories, Burlingame, CA, USA) for 1 h and then in ABC reagent (1:500; Vectastain ABC Elite kit, Vector Laboratories) for 1 h. Subsequently, the labeling was visualized by incubation in 0.02% 3,3-diaminobenzidine (DAB; Sigma), 0.08% nickel (II) sulfate or fluorescein isothiocyanate-tyramide (FITC-tyramide; 1:8000), and 0.001% hydrogen peroxide in PB in Tris-hydrochloride buffer (0.1 M, pH 8.0) for 6 min. DAB-labeled sections were mounted, dehydrated, and coverslipped with Cytoseal 60 (Stephens Scientific). Fluorescently labeled sections were coverslipped with an antifade medium (Prolong Antifade Kit, Molecular Probes, Eugene, OR, USA) following mounting. Pancreas sections were also counterstained with DAPI.

The specificity of this antiserum for amylin in the preoptic area was verified by the same distribution of immunolabeled cells in lactating mother rats as for *in situ* hybridization for amylin, the lack of immunolabeling in the same location in control females, as well as the lack of immunolabeling in the same location as in mother rats using an anti-rat CGRP antiserum, raised against synthetic rat Tyr-CGRP (23–37; cat. no. 1720-9007; Biogenesis, Kingston, NH, USA), and characterized extensively in previous studies (23).

c-Fos immunocytochemistry

Every fourth free-floating section of 5 brains/group (mothers on postpartum d 8 and 9 deprived of pups for 22 h and mothers deprived of pups for 20 h followed by 2 h pup exposure) was immunolabeled as described for amylin immunostaining, except that a rabbit anti-Fos primary antiserum [1:20,000; c-Fos(4) sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used.

For double labeling of amylin and c-Fos, every fourth free-floating section was first immunolabeled for amylin, as described above, using FITC-tyramide amplification. Then, sections were placed in rabbit anti-Fos primary antiserum (1:10,000) for 24 h at room temperature. The sections were then incubated in Alexa 594 donkey anti-rabbit secondary antibody (1:500; Molecular Probes) for 2 h, washed in PB overnight, mounted, and coverslipped.

Double-labeling of c-Fos immunoreactivity and amylin mRNA

Perfusion-fixed, cryoprotected brains of 3 pup-exposed mothers were frozen and sectioned at a thickness of 20 μm using a cryostat. Slide-attached sections were processed for *in situ* hybridization, as described above. Immunolabeling for c-Fos was performed before dipping the slides into autoradiographic emulsion. The immunolabeling protocol was the same as that used for single labeling c-Fos immunocytochemistry, except for the use of the rabbit anti-Fos primary antiserum at a dilution of 1:7000. c-Fos was visualized using nickel-intensified DAB reactions, after which the *in situ* hybridization procedure was continued by dipping the slides into emulsion.

Analysis of amylin and c-Fos double labeling

All sections containing amylin neurons (typically 3 sections/brain) were analyzed. The numbers of amylin-immunoreactive (ir) neurons, as well as the number of cells labeled for both amylin and c-Fos, were counted in the medial preoptic nucleus (MPN), the medial preoptic area (MPA), and the ventral subdivision of the bed nucleus of the stria terminalis (BNSTv) using the ×20 objective of an Olympus BX60 light microscope equipped with fluorescent epi-illumination and a

filter that allowed us to see both green and red colors (Olympus, Tokyo, Japan).

Histological analysis

Sections were examined using an Olympus BX60 light microscope equipped with fluorescent epi-illumination and a dark-field condenser. Images were captured at a 2048- × 2048-pixel resolution with a SPOT Xplorer digital charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI, USA). Confocal images were acquired with a Nikon Eclipse E800 confocal microscope (Nikon, Tokyo, Japan) equipped with a Bio-Rad Radiance 2100 Laser Scanning System using ×20–60 objectives at an optical thickness of 1–3 μm.

The contrast and sharpness of the images were adjusted using the levels and sharpness commands in Adobe Photoshop CS 8.0 (Adobe Systems, San Jose, CA, USA). The full resolution of the images was maintained until the final versions were adjusted to a resolution of 300 dpi.

RESULTS

Time course and distribution of amylin mRNA expression in the peripartum and postpartum periods

A very low level of amylin mRNA was detected on d 21 of pregnancy (**Fig. 1*A***). We found a combined 5.3 ± 1.0 amylin mRNA-expressing cells in one side of the brain. The number of autoradiography grains per cell was 30.8 ± 5.2. This intensity of the amylin mRNA signal was similar to that reported previously in virgin female rats (10). After parturition, a significant increase was detected in both the number of amylin mRNA-expressing neurons (F=60.86), and the number of autoradiography grains per neuron (F=44.82). At 1 d after delivery, a large number of amylin-expressing neurons (81.3±8.7 amylin mRNA-expressing cells in one side of the brain) appeared in the preoptic area (Fig. 1*B*). The autoradiography signal was intense in these labeled neurons (66.5±5.3 grains/cell). At 9 and 23 d after parturition, amylin established an even higher level of expression in the same preoptic brain regions than at 1 d postpartum (Fig. 1*C, D*). The combined numbers of amylin mRNA-expressing cells in one side of 3 consecutive sections were 120 ± 9.5, and 108 ± 3.3, respectively. The numbers of autoradiography grains per cell were 105.6 ± 5.3 and 102.1 ± 7.3, respectively (Fig. 1*E, F*). Amylin-expressing neurons were situated in the MPN, parts of the MPA, and BNSTv at the level of the anterior commissure. Within these regions, amylin mRNA-expressing neurons did not form a compact cluster of cells but were relatively evenly dispersed among other types of neurons. In addition to these preoptic locations, we detected no signal for amylin mRNA in the examined parts (between bregma levels +3 and −3 mm) of the brain in pregnant rats and lactating mothers.

Amylin-ir cell bodies were not detected in the preoptic area on d 21 of pregnancy (**Fig. 2*A***). In contrast, a large number of amylin-ir cell bodies (96.0±33.2/side, 3 consecutive sections, n=5 brains) were observed in the preoptic area of mother rats on d 9 postpartum (Fig. 2*B*). These immunolabeled neuronal perikarya were located in the MPN, parts of the MPA, and the BNSTv (Fig. 2*B*). Thus, the distribution of amylin-ir neurons in the preoptic area of mother rats was the same as that of amylin mRNA-expressing neurons. A similar induction in amylin mRNA was not observed in the pancreas. The intensity of amylin immunoreactivity in pancreatic islets appeared similar in lactating mothers and mothers deprived of her pups immediately after parturition (Supplemental Fig. S1).

Effect of ovariectomy and maternal sensitization on amylin levels in the preoptic area

Amylin mRNA signal in the preoptic area was very low at 2 wk after ovariectomy (**Fig. 3*A***). No induction was detected, as compared to that in control virgin female rats. In contrast, maternal sensitization elicited a robust induction of amylin expression in the preoptic area (Fig. 3*B*). The distribution pattern of amylin-expressing neurons in maternally sensitized rats was the same as that in mother rats (Fig. 3*B*), and labeled neurons were not detected in the brains of sensitized rats outside the preoptic area.

In subsequent quantitative RT-PCR studies, a 17.9 times higher (P<0.03) mRNA level of amylin was found in maternally sensitized virgin female rats, as compared to control virgin female rats (**Fig. 4**). The mRNA level of amylin (expressed as 10^5 mRNA level/mRNA level of GAPDH) was 332 ± 115 for sensitized rats and 18 ± 9 for control female rats, whereas there was no difference in the level of GAPDH mRNA between the 2 groups (638±84 *vs.* 777±272 fg/μl in maternally sensitized and control female rats, respectively).

Amylin-ir neurons were abundant in the preoptic area of ovariectomized and maternally sensitized nulliparous female rats (**Fig. 5*A***). The distribution pattern of amylin-ir neurons was the same in maternally sensitized (Fig. 5*A*) and mother rats (Fig. 5*B*). In contrast, amylin-ir neurons were not detected in any parts of the preoptic area in nulliparous females not sensitized despite being exposed to pups for 8 d (Fig. 5*C*), and in nonsensitized control female rats (Fig. 5*D*).

Pup exposure-induced activation of amylin-expressing neurons in the preoptic area of rat dams

Removal of the pups from the mothers for 22 h resulted in a considerable decrease in the number of c-Fos-positive neurons in the maternal brain. On returning the pups, the dams all took care of them immediately, and suckling started within 10 min. c-Fos-ir neurons appeared in response to pup exposure in a number of brain regions, including parts of the preoptic area, but also in other brain regions, including the anteroventral periventricular nucleus, the lateral septal nucleus, and the periaqueductal gray. Within the preoptic area, c-Fos-ir (Fos-ir) neu-

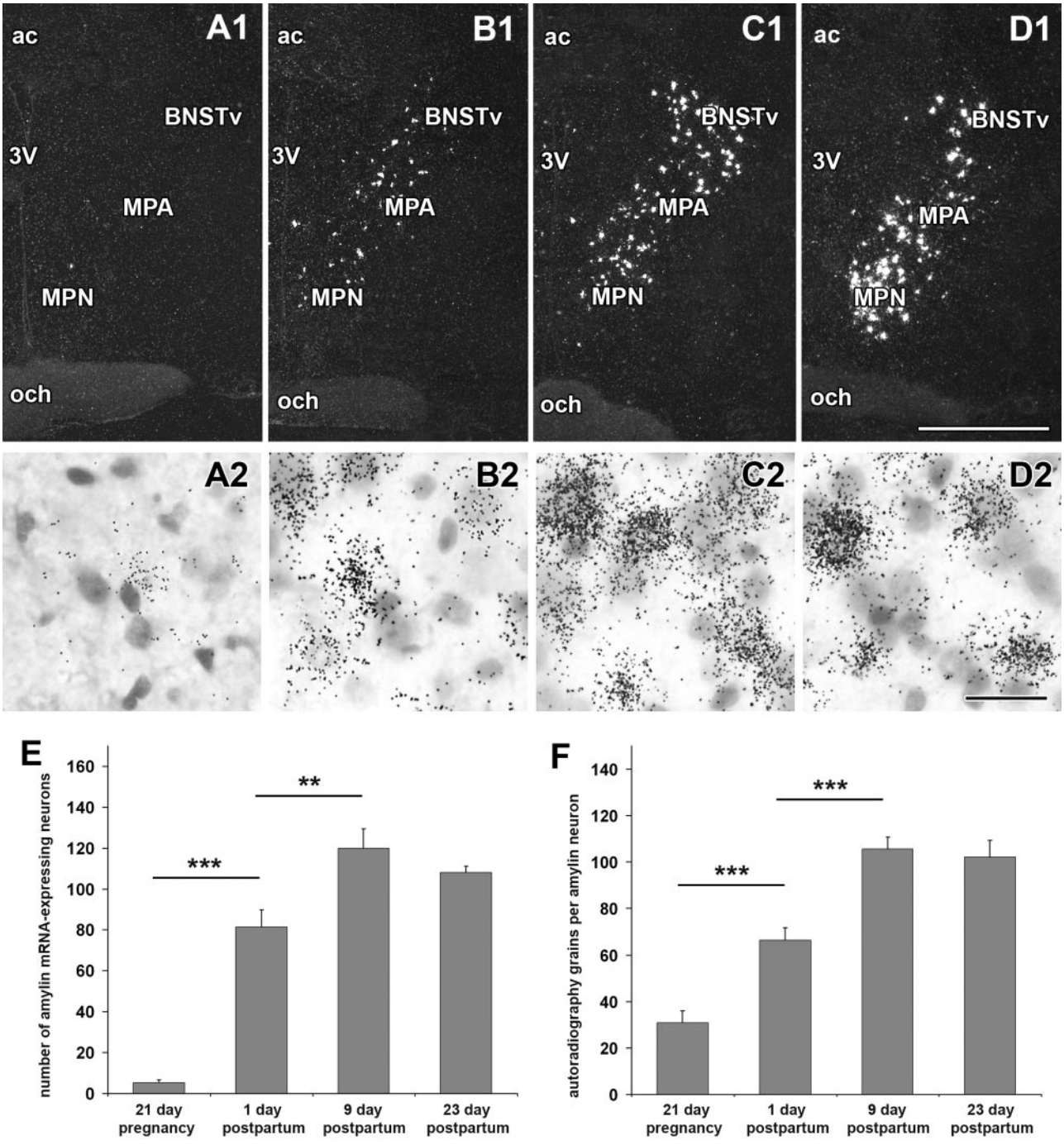

Figure 1. Radioactive *in situ* hybridization histochemistry demonstrates amylin mRNA-expressing neurons in the preoptic area at bregma level, 0.24 mm. *A1–D1*) Distribution of amylin mRNA is shown in dark-field photomicrographs; white signal represents amylin mRNA. Amylin mRNA is distributed in the medial preoptic nucleus (MPN), dorsolateral to the MPN in parts of the medial preoptic area (MPA) and further dorsolateral to this cell group in the ventral part of the bed nucleus of the stria terminalis (BNSTv). *A2–D2*) High-magnification bright-field photomicrographs demonstrate individual autoradiography grains (black dots) above amylin mRNA-expressing preoptic neurons. Amylin mRNA expression is very low on d 21 of pregnancy (*A*) but increases by d 1 postpartum (*B*) and becomes even higher by d 9 (*C*) and d 23 (*D*) postpartum. *E*) Total number of amylin mRNA-expressing neurons counted in the same side of 3 consecutive coronal sections positioned at 216 μm from each other (n=6/time point). A significant increase was found between d 21 of pregnancy and d 1 postpartum. Further significant increase takes place in the number of amylin mRNA-expressing neurons between d 1 and 9 postpartum. *F*) Number of autoradiography grains in randomly selected amylin mRNA-expressing neurons (10 neurons/side of brain). A significant increase was found between d 21 of pregnancy and d 1 postpartum and also between d 1 and 9 postpartum (n=6/time point). Scale bar = 1 mm (*A1–D1*); 30 μm (*A2–D2*). $^{**}P < 0.01$; $^{***}P < 0.001$.

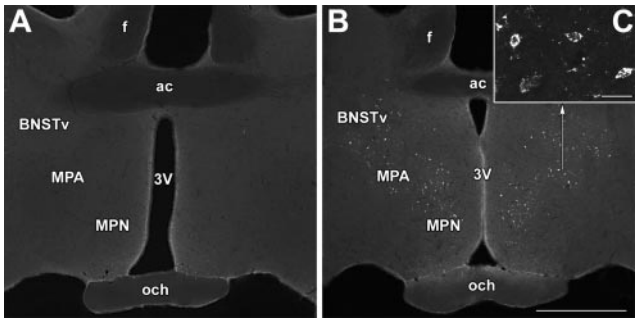

Figure 2. Amylin immunolabeling in the preoptic area of mother rats at bregma level −0.44 mm. *A*) Amylin-ir neurons are not present in the preoptic area of a rat sacrificed on d 21 of pregnancy. *B*) Preoptic area of a mother rat on d 9 postpartum contains intensely immunolabeled amylin-containing cell bodies distributed in the MPN, MPA, and BNSTv. *C*) Confocal image corresponding to area indicated by the white arrow demonstrates immunolabeling of amylin-containing neurons at high amplification. Scale bars = 1 mm (*B*); 50 μm (*C*).

rons appeared (a combined 584±72 in 3 consecutive preoptic sections) in the MPN, MPA, and BNSTv when the dams were exposed to their pups for 2 h following 20 h of separation (**Fig. 6*A***). Thus, the distribution of neurons activated by pup exposure was very similar to the distribution of amylin-expressing neurons. In turn, only a low number of Fos-ir neurons (a combined 71±21 in 3 consecutive sections) were detected in the preoptic area of rat dams 22 h after separating them from their pups (Fig. 6*B*). Fos-ir nuclei were evenly distributed within the region of the preoptic area that contained Fos-ir neurons, whereas adjacent brain areas, including other parts of the preoptic area and the bed nucleus of the stria terminalis, remained almost completely devoid of Fos-ir cells.

The number of amylin-ir neurons with an identifiable nucleus in the preoptic area was a combined 174.2 ± 61.0 in 3 consecutive preoptic sections (n=5 brains). The number of amylin neurons was about equally high in the MPN and MPA and lower in the BNSTv (**Table 1**). Double-immunolabeling revealed that 85.6% of amylin-ir neurons in the preoptic area were c-Fos positive (Fig. 6*C*, *D*). The percentage of c-Fos-positive amylin neurons did not differ between the MPN, MPA, and BNSTv (Table 1). The few amylin-ir neurons whose cell bodies lacked c-Fos immunoreactivity and the c-Fos-positive but amylin-negative neurons did not have a distinct distribution from that of c-Fos-positive amylin neurons.

The coexpression of amylin and *c-fos* genes in the preoptic area of pup-exposed mother rats was also evident based on the double labeling of amylin mRNA and c-Fos immunoreactivity (**Fig. 7**). The number of amylin mRNA-containing neurons was 113 ± 15 in a side of the sections that contained amylin mRNA-expressing neurons. c-Fos immunoreactivity was present in 93.2% of these amylin-expressing neurons.

DISCUSSION

The involvement of amylin in maternal control was studied by several different approaches in this study. In this discussion, we first compare the induction pattern of amylin obtained with different methods. Then, we discuss the activation of amylin neurons and stimuli that may activate these neurons in mothers. Next, we describe current evidence supporting the hypothesis that amylin is neuropeptide with potential modulatory actions in postpartum dams. Finally, the possible neural functions of the brain amylin system are summarized.

Induction of amylin in the preoptic area

The elevated *in situ* hybridization signal of amylin during the postnatal period in rat dams, as well as in maternally sensitized nulliparous rats, suggest an elevated expression level of amylin. This was supported by the quantitative RT-PCR data, which confirmed the induction of amylin mRNA in maternally behaving rats. Our data also provide information on the time course of amylin induction. Amylin mRNA is not induced 1 d before parturition but is already present 1 d after delivery. Amylin immunoreactivity also appears in the postpartum but not in the prepartum period, which confirms the specificity of the amylin signal and suggests that the elevation of amylin mRNA is translated into an increased synthesis of amylin peptide. The number of amylin mRNA-containing and amylin-ir neurons is difficult to compare because the sections for immunoreactivity were thicker (50 *vs.* 12 μm), but the penetration of antiserum into the sections is limited. Nevertheless, the distribution of amylin mRNA-expressing and amylin-ir neurons was the same in the maternal brain. Therefore, we refer to these cells as amylin neurons. The distribution of amylin neurons was widespread within some but not all parts of the preoptic area. Amylin neurons were confined to the MPN, MPA, and BNSTv, where they were relatively evenly distrib-

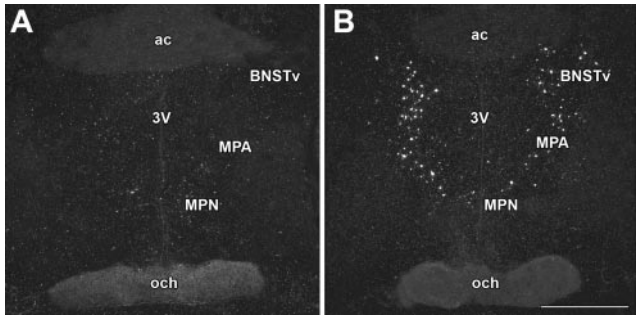

Figure 3. Amylin mRNA expression in the preoptic area of ovariectomized and sensitized nulliparous female rats as demonstrated by dark-field photomicrographs of sections (bregma level: −0.36 mm) labeled with *in situ* hybridization histochemistry. *A*) Ovariectomy did not induce amylin mRNA in any part of the preoptic area. *B*) Maternal sensitization following ovariectomy resulted in an elevated level of amylin mRNA in the MPN, MPA, and BNSTv. Scale bar = 1 mm.

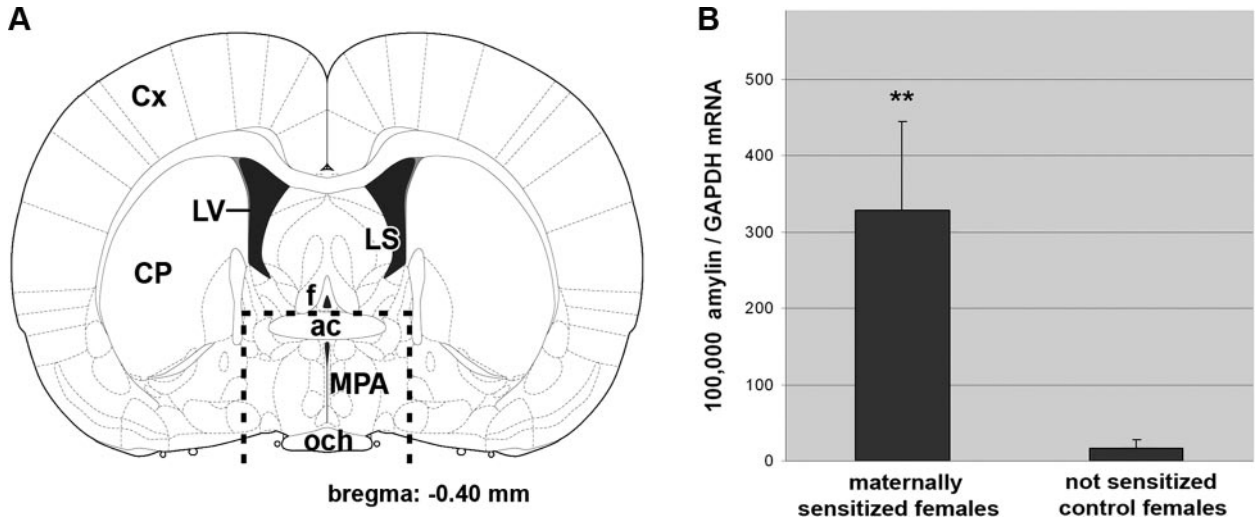

Figure 4. Effects of maternal sensitization on amylin mRNA levels in the preoptic area. *A*) Schematic figure demonstrates the brain region dissected for the quantitative real-time RT-PCR experiment. *B*) Graph shows a significantly increased amylin mRNA level in maternally sensitized virgin female rats as compared to age-matched control female rats. Data are expressed as the ratio of GAPDH mRNA. $^{**}P < 0.03$, $n=8$/group.

uted among other types of neurons. The distribution of amylin neurons in the preoptic area was the same throughout the postpartum period in dams and also in maternally behaving sensitized nulliparous rats, suggesting a common mechanism of induction. Amylin expression remained high as long as the pups were not removed from the mother, suggesting the importance of the presence of pups in amylin induction. In contrast, ovariectomy did not result in an elevated level of amylin mRNA, arguing against a role of sexual steroid hormones in the induction of amylin expression.

Maternal activation of preoptic amylin neurons

c-Fos is an immediate early gene product expressed in activated cells (24). c-Fos appeared in the preoptic area of mother rats in response to pup exposure. c-Fos-expressing neurons were abundant in the MPN, MPA, and BNSTv, and the anteroventral periventricular nucleus. This finding confirmed previously reported expression and distribution patterns of c-Fos in the preoptic area of mother rats (14–16). Furthermore, double labeling of c-Fos with amylin mRNA and amylin immunoreactivity has demonstrated that amylin neurons are essentially all activated by pup exposure, indicating an elevated activity of amylin neurons in postpartum rat dams in this area. This finding also suggests that amylin is induced in the preoptic area of rat dams by the presence of pups. Thus, amylin represents a novel marker of neurons activated in the preoptic area of mothers. Using amylin as a marker, selective investigation of the function of these neurons will be possible using electrophysiological and gene technological procedures.

The type of pup-related stimulus that activates amylin neurons remains undetermined. The suckling stimulus, as a major driver of maternal adaptations, is a leading candidate. Recently, we provided evidence that neu-

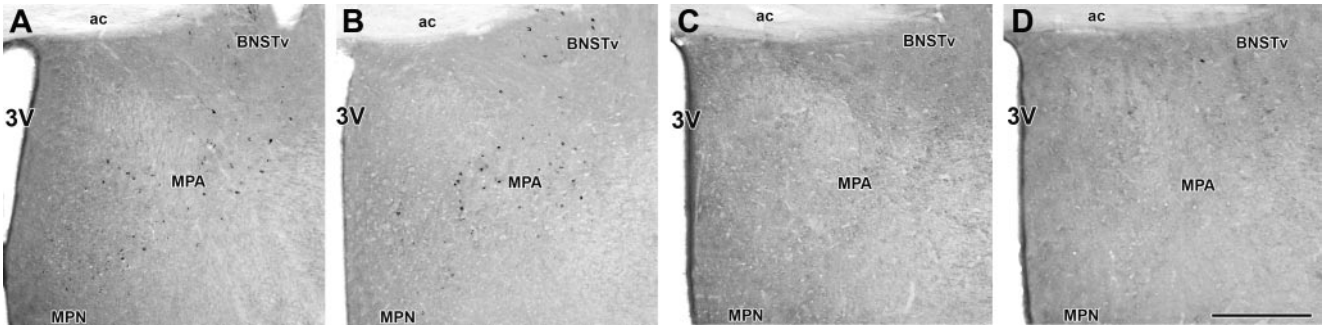

Figure 5. Amylin immunolabeling in the preoptic area of maternally sensitized female rats at bregma level −0.40 mm. *A*) Amylin-ir neurons are present in the MPN, MPA, and BNSTv. *B*) Amylin-ir neurons in the preoptic area of mother rats on d 9 postpartum. Distribution of amylin ir neurons is the same as for maternally sensitized female rats in panel *A*. *C*) Amylin-ir neurons are not present in the preoptic area of nulliparous rats, which did not show maternal sensitization despite having been exposed to foster pups for 8 d. *D*) Amylin-ir neurons are also absent in the preoptic area of nulliparous control female rats not exposed to foster pups. Scale bar = 500 μm.

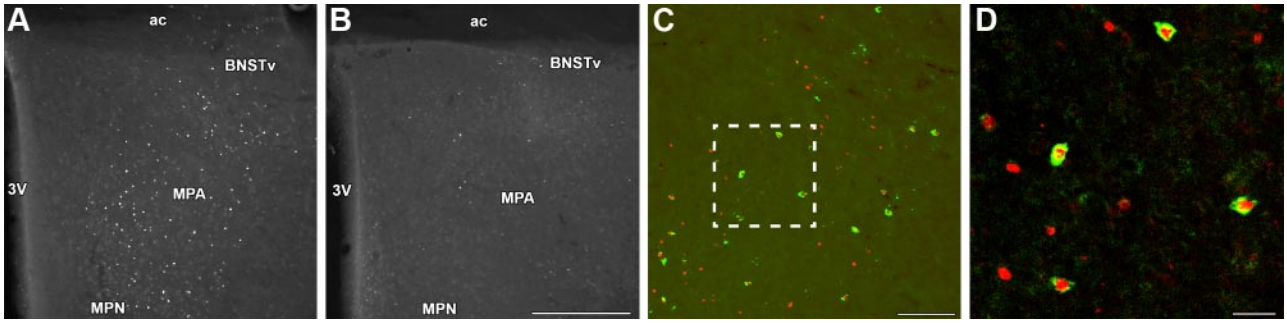

Figure 6. c-Fos activation of amylin-ir neurons in response to pup exposure at bregma level −0.40 mm. *A*) High density of Fos-ir neurons (white dot-like nuclei) was present in the preoptic area of mother rats at 2 h after pup exposure following 22 h of separation. Fos-ir neurons were present in the MPN, MPA, and BNSTv with a distribution similar to that of amylin-expressing neurons. *B*) Number of Fos-ir neurons was very low in the preoptic area of mother rats whose pups were not given back to them. *C*) Most amylin-ir neurons (green cell bodies) contain c-Fos (red stained nuclei) in the preoptic area following pup exposure, as demonstrated by double-fluorescent immunolabeling. A considerable number of Fos-ir but amylin-negative neurons, distributed evenly among double-labeled cells, are also visible. *D*) Confocal photomicrograph shows neurons labeled for amylin and c-Fos at high magnification. Numbers of amylin-ir neurons with identifiable cell nuclei and percentages of Fos-ir amylin neurons are quantified in Table 1. Scale bars = 500 μm (*B*); 200 μm (*C*); 50 μm (*D*).

rons in the posterior intralaminar complex of the thalamus relay the suckling information toward medial hypothalamic sites that regulate prolactin release (25). This thalamic region projects to the preoptic area (26); therefore, activation of amylin neurons could occur *via* posterior thalamic neurons relaying suckling information (27). Neuronal tract tracing methods revealed a number of additional inputs to the preoptic area (28). Neurons in some of the input regions of the preoptic area are activated in the presence of pups (29, 30) and may convey visual, auditory, or olfactory exteroceptive stimuli associated with the presence of pups, which could contribute to the activation of amylin neurons in response to pup exposure.

Amylin as a novel neuropeptide potentially involved in maternal control

The present study provided evidence that amylin appears in the brain of maternally behaving rats, which makes amylin a neuropeptide candidate. Amylin is known to be stored (Supplemental Fig. S1) and released from vesicles in the pancreas (5); therefore, it is likely that amylin also possesses a regulated vesicular release from terminals of preoptic neurons. Neurons in the medial preoptic nucleus, and surrounding regions activated by pup exposure project to different brain regions, including the lateral septum, the bed nucleus of the stria terminalis, the substantia innominata, the amygdala, and several different parts of the hypothalamus, including the periparaventricular zone, the ventromedial and arcuate nuclei, and the lateral hypothalamic area, as well as the periaqueductal gray (16, 31). Some of these projections may contain amylin in maternally behaving rats, which could activate amylin receptors present in these target areas (32–34). Therefore, amylin, together with its receptors, is a candidate to form a peptide neuromodulator system in the maternal brain. Thus, amylin can be considered along with

TABLE 1. *Number of amylin-ir neurons with identifiable cell nucleus and percentage of Fos-ir amylin neurons in the MPN, MPA, and BNSTv*

Parameter	MPN	MPA	BNSTv
Amylin neurons with nucleus (*n*)	65.0 ± 30.7	70.0 ± 29.2	39.2 ± 23.7
Fos-ir amylin neurons (%)	85.9 ± 2.1	82.9 ± 3.7	90.4 ± 5.2

Amylin neurons were present in 3 coronal sections cut at 200-μm distances (Fig. 6). All amylin-ir neurons were counted in all 3 sections of 5 rats exposed to pups. Values represent average sums (n=5 brains) of labeled cells in the 3 sections that contained amylin-ir neurons.

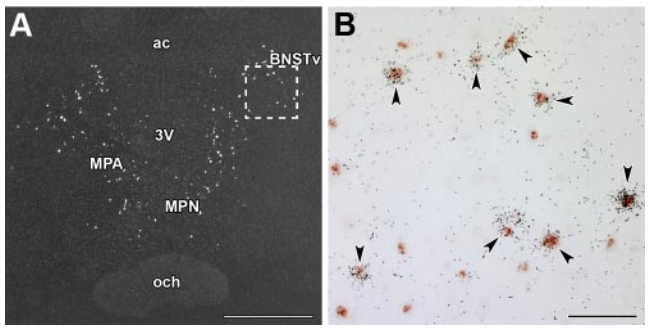

Figure 7. Fos activation of amylin mRNA-expressing neurons in response to pup exposure. *A*) High density of amylin mRNA-expressing neurons (white labeling) can be seen in a dark-field photomicrograph of the preoptic area in mother rat at bregma level −0.4 mm. *B*) Framed area in A is shown in a bright-field image at high magnification. Most amylin mRNA-expressing neurons (situated below the black autoradiography dots) contained c-Fos-immunoreactivity (brown nuclei) in the preoptic area at 2 h after pup exposure following 22 h of separation. A considerable number of Fos-ir but amylin-negative neurons are also visible. Fos-ir neurons were present in the MPN, MPA, and BNSTv with a distribution similar to that of amylin-expressing neurons. Scale bars = 1 mm (*A*); 100 μm (*B*).

a number of peptide neuromodulators recognized to be involved in aspects of maternal behaviors, including prolactin, oxytocin, vasopressin, opioids, tachykinins, and corticotropin-releasing hormone (35–37). Even though a significant increase has been reported in the average soma size of preoptic neurons during late pregnancy and lactation, suggesting increased gene expressional activity (38), prolactin, oxytocin, and vasopressin are not expressed in the preoptic area even in mothers. Nevertheless, these peptides released from fibers terminating in the preoptic area may affect maternal behaviors as recently evidenced for vasopressin (39). Other neuropeptides, such as opioids, tachykinins, and corticotropin-releasing hormone, are expressed in the preoptic area (40). These peptides are, however, expressed in a number of additional brain regions, too. Thus, the restricted distribution of amylin expression in the preoptic area is unique among neuropeptides.

Potential neural functions of preoptic amylin

The marked elevation of the level of amylin and the activation of amylin neurons in response to pup exposure suggest that amylin is involved in some aspects of maternal control. Lactation represents a heavy metabolic load for the mothers, who lose weight during this period (41, 42). Adiposity was suggested to be regulated by specific maternal mechanisms other than the homeostatic regulations taking place throughout the life span (43). Because the activation of brain amylin receptors has been suggested to decrease body adiposity in rats (44), it is conceivable that maternal amylin plays a role in the regulation of body weight in dams. However, it is likely that maternal actions of central amylin are not related to the actions of peripheral amylin because the anorectic actions of peripheral amylin are completely eliminated by lesions of the dorsal vagal complex (45).

Bilateral lesion of the preoptic area completely eliminates maternal behaviors (12, 13); therefore, amylin induced in this region could be involved in the regulation of maternal behaviors. Furthermore, maternally sensitized nulliparous females do not lactate, but they do demonstrate maternal behaviors, including nest building, pup retrieval, and anogenital licking (17, 18). We found that c-Fos is activated in the preoptic area of these animals in response to pup exposure and that amylin neurons are among those activated in the region. Maternally activated preoptic neurons also project to brain regions involved in maternal regulations (46). Some of these brain regions express amylin receptors, including the bed nucleus of the stria terminalis, various hypothalamic and amygdaloid nuclei, and the periaqueductal gray (32–34). Consequently, a likely hypothesis is that amylin plays a role in the regulation of some of the maternal behaviors that appear during the postpartum period.

In addition to metabolic and behavioral changes, endocrine and emotional adaptations take place in mothers for a limited period of time to support the offspring (47). These include lactation driven by prolactin and oxytocin release (48, 49), lactational anoestrous by the suppression of GnRH secretion (50), maternal aggression toward intruders, decreased anxiety, and reduced responsiveness of the hypothalamo-pituitary-adrenal axis (51). Amylin, induced in the postpartum period, could be involved in the control of emotional and endocrine alterations in mothers. Consequently, it represents a new therapeutic direction to treat dysfunctions associated with motherhood, including postnatal depression (52).

In summary, we report an increased expression of amylin in the preoptic area of postpartum but not prepartum rats. Amylin expression was also elevated in maternally sensitized nonlactating nulliparous female rats, but not in response to ovariectomy, suggesting the importance of the presence of pups in the induction of amylin. Furthermore, amylin neurons demonstrated c-Fos activation by pup exposure. These expressional data and correlative functional evidence suggest that amylin is a neuropeptide, which might be involved in the physiological regulation of some aspects of maternal adaptations. FJ

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