Data Supporting a New Physiological Role for Brain Apelin in the Regulation of Hypothalamic Oxytocin Neurons in Lactating Rats

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Apelin is a bioactive peptide identified as the endogenous ligand of the human orphan G protein-coupled receptor APJ in 1998. The present data show that apelin modulates the activity of magnocellular and parvocellular oxytocin (OXY) neurons in the lactating rat. A combination of *in situ* hybridization and immunohistochemistry demonstrated the presence of apelin receptor mRNA in hypothalamic OXY neurons. Double immunofluorescence labeling then revealed the colocalization of apelin with OXY in about 20% of the hypothalamic OXY-positive neurons. Intracerebroventricular apelin administration inhibited the activity of magnocellular and parvocellular OXY neurons, as shown by measuring the *c-fos* expression in OXY neurons or by direct electrophysiological measurements of the electrical activity of these neurons. This effect was correlated with a decrease in the amount of milk ejected. Thus, apelin inhibits the activity of OXY neurons through a direct action on apelin receptors expressed by these neurons in an autocrine and paracrine manner. In conclusion, these findings highlight the inhibitory role of apelin as an autocrine/paracrine peptide acting on OXY neurons during breastfeeding. **(*Endocrinology* 152: 3492–3503, 2011)**

Apelin 36 is a bioactive peptide that has been identified as the endogenous ligand of the human orphan G protein-coupled receptor APJ (1, 2). APJ is now therefore commonly referred to as the apelin receptor (3). Apelin 36 is generated from a single 77-amino acid precursor, preproapelin, for which cDNA have been cloned from humans, cattle, rats, and mice (2, 4–6). An alignment of these amino acid sequences showed that the C-terminal sequence between Trp55 and Phe77, including the C-terminal 17 (Lys-Phe-Arg-Arg-Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe) [apelin 17 fragment (K17F)] and 13 (Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe; Q13F) amino acid sequences was fully conserved (2, 4, 6). The glutamine residue at the N terminus of Q13F may be pyroglutamylated to generate the pyroglutamyl form of apelin 13 (pE13F). Both peptides (K17F or pE13F) naturally occur in rat brain and plasma (7, 8). K17F has an affinity (Ki, 0.04 nM) 10 times higher than that of pE13F

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Abbreviations: ABC, Avidin-biotin peroxidase complex; Acc, accessory neurosecretory nuclei; aCSF, artificial cerebrospinal fluid; AVP, arginine vasopressin; icv, intracerebroventricular; K17F, apelin 17 fragment; ME, median eminence; NTS, nucleus of the solitary tract; OXY, oxytocinergic; PaAP, anterior parvocellular part of the PVN; PaDC, dorsal cap of the PVN; PaLM, lateral magnocellular part of the PVN; PaMP, medial parvocellular part of the PVN; PaPo, posterior part of the PVN; PaV, ventral part of the PVN; Pe, periventricular hypothalamic nucleus; pE13F, pyroglutamyl form of apelin 13; PRL, prolactin; PVN, paraventricular hypothalamic nucleus; SO, more rostral part of the SON; SON, supraoptic hypothalamic nucleus; SOR, retrochiasmatic part of the SON; TH, tyrosine hydroxylase.

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(Ki, 0.4 nM) for the human or rat apelin receptor (9). K17F and pE13F have similar inhibitory effects (IC_{50}: 0.08 and 0.2 nM, respectively) on forskolin-induced cAMP production in CHO cells stably expressing the rat apelin receptor (9), but K17F induces internalization of the rat apelin receptor more efficiently than pE13F (10). Moreover, *in vivo*, both peptides have robust pharmacological effects when injected intracerebroventricularly (icv) into rodents (11). Consequently, most studies investigating the involvement of apelinergic systems in various functions have focused on K17F and pE13F (8, 11–17).

Apelin and its receptor are widely distributed throughout the rat nervous system (6, 8, 11, 12, 18–21) and are particularly strongly expressed in the supraoptic hypothalamic nucleus (SON) and paraventricular hypothalamic nucleus (PVN) (8, 11, 12, 18). Consistent with this distribution, there is growing evidence to suggest that apelin and its receptor play an important role in the control of body fluid homeostasis, by interacting with vasopressinergic systems: 1) apelin and its receptor colocalize with arginine vasopressin (AVP) in the SON and PVN magnocellular neurons (8, 11, 12); 2) when administered icv to lactating rats, K17F inhibits the phasic electrical activity of magnocellular AVP neurons, thereby decreasing the release of AVP into the bloodstream and increasing aqueous diuresis (8); 3) in water-deprived rats, endogenous levels of AVP and apelin are regulated in opposite manners, to optimize the systemic AVP release required to avoid additional water loss in the kidney (8, 12); and 4) plasma apelin and AVP levels are also regulated in opposite manners, by osmotic stimuli, in humans (7). All these studies suggested that apelin, like AVP, may participate in the maintenance of body fluid homeostasis not only in rodents but also in humans.

It has also been reported that apelin interacts with the hypothalamic oxytocinergic (OXY) neurons. Firstly, dual-labeling studies in male and virgin female rats have shown that SON and PVN magnocellular OXY neurons also contain apelin (22). Secondly, in the SON, which is known to comprise mostly AVP and OXY neurons (23), both AVP and non-AVP neurons produce apelin receptor mRNA (11), suggesting that OXY neurons may also express this receptor. These data are consistent with physiological relevance of the similar distributions of apelin, apelin receptors, and OXY neurons. OXY is involved in many physiological functions, including parturition, lactation, and maternal behavior in females, the erection and ejaculation processes in males, and the maintenance of water and mineral balance in the body, together with social behavior, in animals of both sexes (24, 25). However, the only nonredundant role of OXY is in the control of milk release: OXY-deficient mice cannot feed their young, as no milk is released when the pups suckle (25, 26). The stimulation of mechanoreceptors in the nipple by suckling pups initiates the milk ejection reflex. The ascendant nervous system route responsible for transmitting this information through the spinal cord and forward to the hypothalamus is not fully understood. However, noradrenaline, released in the PVN and SON from noradrenergic neurons originating from the nucleus of the solitary tract (NTS) (the A2 region), is widely thought to be the best candidate final mediator of the suckling-induced activation of OXY cells (25, 27, 28). Activated magnocellular OXY neurons release OXY into the bloodstream, where it acts on myoepithelial cells of the mammary gland to cause contraction of the smooth muscle (see for review Ref. 25). In addition, OXY released from the parvocellular PVN OXY neurons in the portal circulation plays a role in milk production, by increasing the prolactin (PRL) production of lactotrophs in the anterior pituitary gland (29–31).

In this study, we investigated interactions between apelin and OXY in the lactating rat. We first investigated whether apelin and its receptor colocalized with OXY in hypothalamic OXY neurons. In parallel, we investigated whether apelin colocalized with tyrosine hydroxylase (TH) in the NTS noradrenergic neurons known to control magnocellular OXY neuron activity during milk ejection. We then assessed the possible impairment of OXY neuron activity by apelin, by determining the effect of K17F administered icv on both *c-fos* expression in all hypothalamic OXY neurons and the electrical activity of SON magnocellular OXY neurons. Finally, we investigated whether apelin modified the amount of milk delivered to littermates, by impairing the activity of OXY neurons, thereby inhibiting systemic OXY release.

Materials and Methods

Drugs and antibodies

K17F (Lys-Phe-Arg-Arg-Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe) was synthesized by Neosystem (Strasbourg, France). Rabbit polyclonal antibodies directed against K17F were produced in the laboratory and characterized in previous studies (8, 11, 18). We also used a monoclonal mouse anti-OXY antibody (PS-38; American Type Culture Collection, Manassas, VA) (32), a polyclonal rabbit anti-c-Fos antibody (sc-52; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (33), and a monoclonal mouse anti-TH antibody (22941; DiaSorin, Stillwater, MN) (34). Colchicine was obtained from Sigma-Aldrich (St. Quentin, France).

Animals

Lactating Wistar rats (303 ± 11 g body weight) were purchased from Charles River Laboratories (L'Arbresle, France). They were provided with free access to food and water and maintained on a 12-h light, 12-h dark cycle. All experiments were

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carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Distribution of apelin immunoreactive neurons in the brain stem and hypothalamic regions

Intracerebroventricular colchicine injection

Lactating rats (n = 5) were used for the dual detection of apelin/OXY or apelin/TH by immunofluorescence on postnatal d 10–11. On postnatal d 8–9, rats were anesthetized by ip injection of pentobarbital (Nembutal; 50 mg/kg) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) for implantation of a cannula into the right lateral ventricle (bregma, 1 mm caudal, 1.5 mm lateral, depth 4 mm). Colchicine [100 μg in 5 μl of artificial cerebrospinal fluid (aCSF): 125 mM NaCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 27.0 mM $NaHCO_3$, and 0.5 mM NaH_2PO_4)] was immediately injected icv, to increase the concentration of apelin in the neuronal somata (18). Animals were maintained for 2 d in the animal room, under frequent observation, and then anesthetized and fixed as described in the Supplemental Materials and Methods, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Dual immunofluorescence labeling for apelin and OXY or apelin and TH

Dual detection of apelin and OXY was carried out on 30-μm-thick free-floating sections from the anterior hypothalamus, and dual detection of apelin and TH was carried out on 30-μm-thick free-floating sections from the brain stem, as detailed in the Supplemental Materials and Methods. We processed every third section for immunofluorescence procedures. Sections were first incubated with the primary antibody against K17F (1:1000; 48–72 h, 4 C), then with a biotinylated secondary antibody and avidin-biotin peroxidase complex (ABC), and finally with Alexa Fluor 647-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Anterior hypothalamic sections were then incubated with the OXY antibody (1:75,000), whereas brain stem sections were incubated with the TH antibody (1:4000). OXY and TH immunoreactivities were then detected by incubating the section with an Alexa Fluor 488-labeled goat antimouse antibody (Molecular Probes, Eugene, OR). Control sections were processed in parallel but with the omission of primary or secondary antibodies. In addition, as described in the Supplemental Materials and Methods, the specificity of the primary antibody against K17F was checked by reacting hypothalamic sections from lactating rats, in parallel, with the antibody against K17F with or without before incubation of the antibody with 25 μg/ml K17F for 30 min at room temperature. No immunostaining was observed if the antibody against K17F was preincubated with K17F (Supplemental Fig. 1A), whereas typical staining of apelin-positive somata was observed in the absence of before incubation of the antibody with K17F (Supplemental Fig. 1B). Sections were prepared for observations under a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany), consisting of a DMI6000 inverted microscope equipped with an argon laser (488 nm, 200 mW) and a helium laser (647 nm, 15 mW), with ×10, ×40, and ×63 oil immersion objectives. Sections were scanned, and double-labeling images were obtained with two photomultipliers and appropriate filter settings for the separate detection of fluorescence from Alexa Fluor 488 (500–550, green) and Alexa Fluor 647 (650–750, red). Confocal images were obtained as LIF files from Leica Confocal Software (Leica application suite advanced fluorescence). Two investigators counted apelin- and OXY-positive neurons at ×630 magnification, on one entire side of the neuraxis according to the atlas of Paxinos and Watson (35). The number of sections analyzed for a defined structure (or subnucleus) therefore depended on the structure concerned. In a defined structure (or subnucleus), the values obtained by the two investigators were averaged for each section analyzed. The mean numbers of positive neurons per section and per structure (or subnucleus) are expressed as means ± SEM.

Simultaneous detection of OXY by immunohistochemistry and of apelin receptor mRNA by in situ hybridization

We obtained 7-μm-thick coronal sections of the anterior hypothalamus from lactating rats (n = 2; postnatal d 10–11), as detailed in the Supplemental Materials and Methods.

TABLE 1. Mean number of neurons per section and per structure or subnucleus apelin-positive, OXY-positive, and apelin-positive/OXY-positive in hypothalamic neuronal groups

	Number of apelin-positive neurons	Number of OXY-positive neurons	Number of apelin-positive and OXY-positive neurons
Magnocellular groups			
Acc	4.4 ± 0.04	9.4 ± 0.4	1.9 ± 0.1
PaLM	5.7 ± 0.4	38.9 ± 0.9	2.0 ± 0.1
SO	3.3 ± 0.3	42.8 ± 0.7	2.0 ± 0.1
SOR	1.9 ± 0.1	12.7 ± 0.3	1.1 ± 0.05
Parvocellular groups			
PaAP	10.6 ± 2.0	41.3 ± 5.2	4.5 ± 0.9
PaDC	2.8 ± 0.2	14.9 ± 0.4	1.0 ± 0.1
PaMP	3.1 ± 0.4	33.5 ± 4.6	0.9 ± 0.1
PaPo	1.6 ± 0.2	6.9 ± 0.1	0.6 ± 0.3
PaV	8.2 ± 0.8	40.7 ± 3.9	1.7 ± 0.2
Magnocellular and parvocellular group			
Pe	0.7 ± 0.01	6.1 ± 0.04	0.2 ± 0.004

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Synthesis of cRNA probes for the rat apelin receptor

Apelin receptor antisense and sense cRNA probes were synthesized by *in vitro* transcription, as previously described (3). Briefly, clone 16.3, which contained the full-length rat apelin receptor cDNA, was digested with *Sac*I and *Eco*RI, and the resulting fragment was inserted between the *Sac*I and *Eco*RI sites of the Bluescript KS plasmid (Stratagene, La Jolla, CA). This plasmid was then used to synthesize the sense and antisense riboprobes. The cDNA was linearized by digestion with *Sac*I or *Xho*I, and *in vitro* transcription was performed with T_3 or T_7 RNA polymerase (Roche Diagnostics, Meylan, France) in the presence of ^{35}S-UTP (PerkinElmer, Courtaboeuf, France). Transcription yield was checked with a β-particle counter.

In situ *hybridization and immunohistochemistry for OXY*

Sections were hybridized with the ^{35}S-labeled apelin receptor antisense and sense cRNA probes (see technical details in Ref. 36). Briefly, after treatment with proteinase K (5 μg/ml for 10 min), each section was incubated overnight with 80 μl of hybridization mixture containing 10.6×10^5 cpm of the sense or antisense probe, in a humid chamber, at 50 C. As detailed in the Supplemental Materials and Methods, the sections were then thoroughly washed and incubated with an OXY antibody (1:75,000; 45 min) and then with a biotinylated secondary antibody and ABC; the corresponding peroxidase activity was detected by incubation with DAB+ (Dako, Trappes, France). The sections were immersed in Kodak NTB2 liquid emulsion for 10 wk (Kodak, Strasbourg, France) and were then counterstained with toluidine blue and prepared for observations under a Leica DM 4000B microscope and photographied with Leica Application Suite software. Structures and subnuclei were delineated according to the atlas of Paxinos and Watson (35). We measured the density of black grains in OXY-positive cells and in areas not containing OXY-positive cells and compared the values obtained in paired Student's *t* tests. Differences were considered significant if $P < 0.05$.

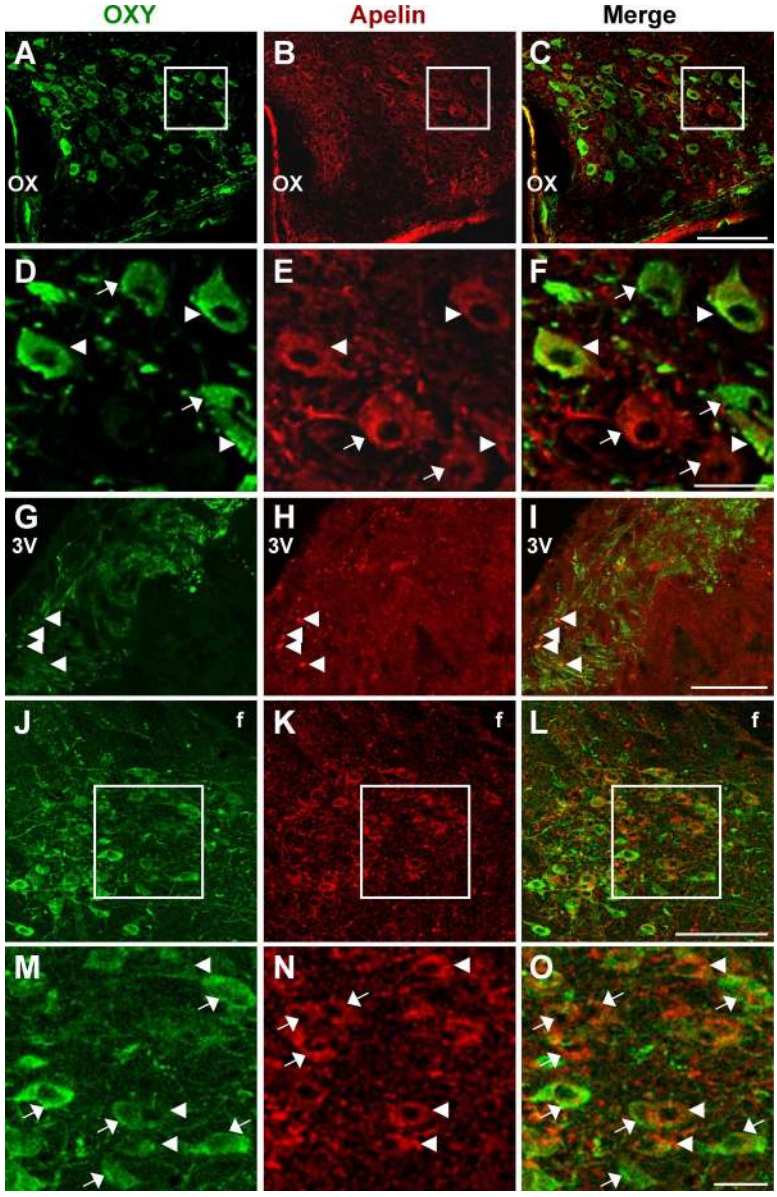

FIG. 1. Distribution of apelin and OXY immunoreactivity in the SON, internal zone of the median eminence, and PaAP in lactating rats. Confocal microscopy images of sections from the SON (A–F), IME (G–I), and PaAP (J–O) double-labeled with antisera against OXY (*green*) and apelin (*red*), in lactating rats. Photomicrographs in D–F correspond to the *white square* in A–C, and those in M–O correspond to the *white square* in J–L. Double-labeled cells or thin varicose immunoreactive axons are indicated by *arrowheads*, whereas apelin- or OXY-labeled cells are indicated by *arrows*. *Scale bars*: 100 μm (A–C and J–L), 25 μm (D–F and M–O), and 50 μm (G–I). OX, Optic chiasma; IME, internal zone of the median eminence; 3V, third ventricle; f, fornix.

Analysis of the effect of K17F on OXY hypothalamic neurons in the lactating rat by double-labeling for c-Fos and OXY

Experimental design and K17F icv injection

Lactating rats (n = 17) were used for the immunohistochemical detection of c-Fos and OXY, to evaluate the effect of K17F. Analysis of the expression of *c-fos*, a marker of neuronal activity, is classically used to evaluate changes in neuronal population activity (37). On postnatal d 3, a cannula was implanted into the right ventricle (as described above). The animals were then allowed to recover for 3–4 d, to prevent nonspecific effects due to the surgical procedure. Because *c-fos* expression is a short-term marker of neuronal activity (37), experimental designs have been developed for enhancing the *c-fos* expression linked to suckle by the pups: a nonsuckling period of 4–6 h followed by an additional suckling period (38, 39). In accordance with such experimental protocols, the pups were separated from their mothers for 6 h and then returned for suckling. Ten minutes before the return of litters, the lactating rats received an icv injection of aCSF (5 μl; n = 8) or aCSF supplemented with K17F (10 μg = 4.7 nmol in 5 μl of aCSF; n = 9). The pups were left to suckle for 1 h, and the rats were then anesthetized and fixed, as detailed in the Supplemental Materials and Methods. Separation of the litters from the mother may cause stress. We therefore habituated the rats to 6-h periods of separation, during the 3 d preceding the icv injection. The effect of

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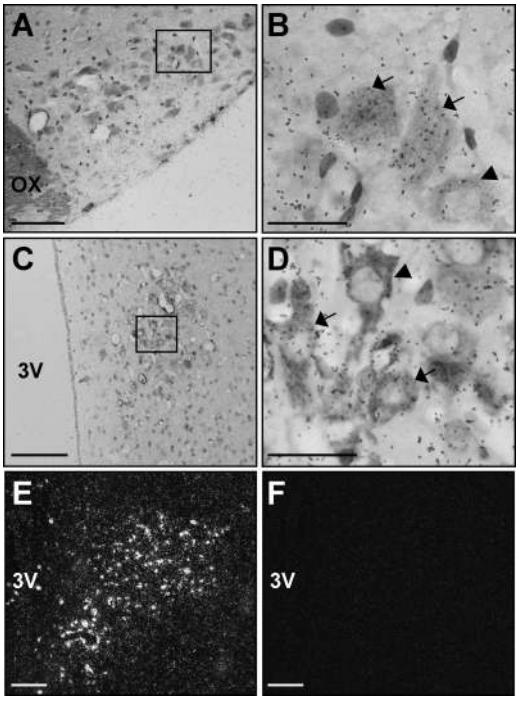

FIG. 2. Simultaneous detection of OXY immunoreactivity and apelin receptor mRNA in the SON and PaAP in lactating rats. The presence of apelin receptor mRNA is indicated by the accumulation of silver grains throughout the cytoplasm of magnocellular OXY neurons in the SON (B, high magnification of the *black square* in A) and of parvocellular OXY neurons in the PaAP (D, high magnification of the *black square* in C). Dark-field photomicrographs showing the labeling of the PVN obtained with apelin receptor antisense (E) and sense (F) riboprobes. *Scale bars*: 100 μm (A and C), 25 μm (B and D), and 100 μm (E and F). OX, Optic chiasma; 3V, third ventricle.

K17F on hypothalamic OXY neurons was therefore assessed on postnatal d 10–11 (as for apelin distribution).

In addition, lactating rats subjected to the same surgical procedure and receiving icv injections, from whom the pups were removed but not returned for a 1-h period of suckling (n = 3), were anesthetized and fixed as reported in the Supplemental Materials and Methods and illustrated in Supplemental Fig. 2.

Dual immunohistochemical labeling for c-Fos and OXY

Sequential labeling was carried out on 30-μm-thick free-floating sections, as detailed in the Supplemental Materials and Methods. We processed every third section for immunohistochemistry procedures. Sections were first incubated with the primary anti-c-Fos antibody (1:4000; 48 h, 4 C), then with a biotinylated secondary antibody and ABC; the corresponding peroxidase activity was detected by incubation with 0.015% 3,3′-diaminobenzidine tetrahydrochloride, 0.04% nickel ammonium sulfate, and 0.006% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). The sections were then incubated with the OXY antibody (1:75,000; 24 h, 4 C), followed by a biotinylated secondary antibody and ABC; the corresponding peroxidase activity was detected by incubation with NOVA RED (Vector Laboratories, Burlington, Canada). Control sections were processed in parallel, but with the omission of the primary or secondary antibodies. Sections were prepared for observations under a Leica DM 4000B microscope (Leica Microsystems) and photographed with Leica Application Suite software. We counted c-Fos- and c-Fos/OXY-positive neurons at ×400 magnification, on one entire side of the neuraxis, according to the atlas of Paxinos and Watson (35). The number of sections analyzed for a defined structure (or subnucleus) therefore depended on the structure concerned. Sections were examined blind, without the observer knowing whether they were from the group of lactating rats receiving icv injections of aCSF alone or from the group of lactating rats receiving icv injections of K17F. The mean numbers of positive neurons per section and per structure (or subnucleus) are expressed as means ± SEM. Differences between the mean numbers of positive neurons per section and per structure between rats receiving icv injections of aCSF alone and rats receiving icv injections of aCSF/K17F were analyzed with unpaired Student's *t* tests. Differences were considered significant if $P < 0.05$.

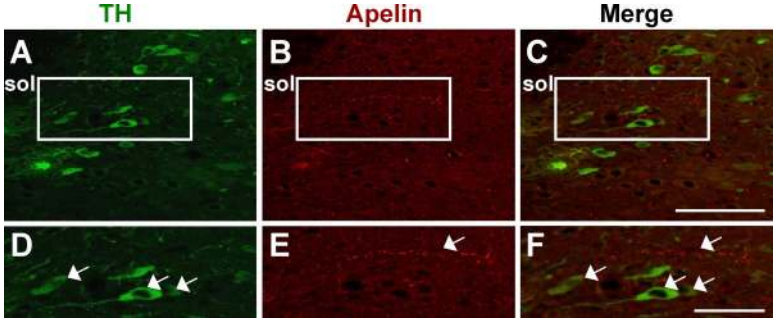

FIG. 3. Apelin and TH immunoreactivities within the NTS/A2 region in lactating rats. Confocal microscopy images of sections from the NTS/A2 region double-labeled with antisera against TH (*green*) and apelin (*red*) in lactating rats (A–F). Photomicrographs in D–F correspond to the *white square* in A–C; *arrows* indicate either apelin-labeled fibers or TH-labeled cells. *Scale bars*, 100 μm (A–C) and 50 μm (D–F). sol, Solitary tract.

Electrophysiological recordings of SON OXY neurons

The procedure used for electrophysiological recordings of OXY neurons in the SON of anesthetized lactating rats (postnatal d 8–12) was as previously described (40). Briefly, dams were anesthetized with urethane (ethyl carbamate; 1.25 g/kg ip). The jugular vein was cannulated for the purposes of drug injection. The head was fixed in a stereotaxic apparatus (and the skull opened), and a stimulating electrode was then inserted into the neural stalk for the antidromic identification of magnocellular neurons. A microsyringe was inserted into the third ventricle (bregma: 8 mm rostral; 0 mm lateral; 3 mm depth; atlas of Paxinos and Watson, see Ref. 35). Two hours after the surgical procedure, a glass microelectrode (filled with 0.5 M sodium acetate) was positioned within the SON, and electrical activity was recorded in 10 pups during suckling. OXY neurons were identified on the basis of their continuous basal activity and their burst of activity in response to suckling. However, suckling did not systematically induce a burst of activity. In the absence of a burst of activity, the OXY nature of the neurons recorded was determined by administering iv injections of cholecystokinin-8 (20 μg/kg; Sigma-Aldrich, St. Louis, MO), which has been shown to excite OXY neurons specifically and transiently (41). After 20 min of recording under basal conditions (control period), K17F (4.3 ng = 2 pmol in 2 μl of aCSF) was injected into the third ventricle.

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Measurement of the amount of milk ingested by a litter

These experiments were carried out on litters from dams included in the dual-immunohistochemistry analysis of c-Fos and OXY. On postnatal d 10–11, pups were separated from their mothers for 6 h, and the litter was weighed before being returned to the mother (control mass). Ten minutes before the return of the litters, lactating rats received an icv injection of either aCSF alone (5 μl; n = 8) or aCSF supplemented with K17F (10 μg = 4.7 nmol in 5 μl of aCSF; n = 9). When the litter was returned to the dams, the time taken by the mother to approach and sniff the pups, to retrieve all the pups, and to position herself over the pups with splayed legs to accommodate them was measured. One hour after the return of the litters, they were weighed again (test mass). The difference between the control and test masses for each litter was used to determine the amount of milk ingested over the 1-h period, as previously described (42). The amount of milk ingested was compared between litters from dams receiving aCSF and from dams receiving aCSF/K17F, in unpaired Student's *t* test. Differences were considered significant if the $P \leq 0.05$.

Results

Distribution of apelin and OXY immunoreactivities in OXY-containing hypothalamic nuclei

This analysis was carried out in the SON and accessory neurosecretory nuclei (Acc) containing only magnocellular neurons (projecting to the neurohypophysis), in the PVN, in which we discriminated between subnuclei containing magnocellular neurons and those containing parvocellular neurons, and in the periventricular hypothalamic nucleus (Pe). The delineation of structures and subnuclei was based on the anatomic location of the groups of neurons studied, according to the atlas of Paxinos and Watson (35).

The mean number of OXY-positive neurons was generally high but differed between the structures studied, whereas the mean number of apelin-positive neurons was small regardless of the structure considered (Table 1). Superimposition of the fluorescence images obtained for apelin and OXY made it possible to determine the number of apelin-positive and OXY-positive neurons regardless of the type of neuron considered: magnocellular (Table 1 and Fig. 1, A–F) or parvocellular (Table 1 and Fig. 1, J–O). Double-labeled neurons accounted for 3–20% of all OXY-positive neurons and 21–61% of all apelin-positive neurons. An analysis of labeling for the median eminence (ME) confirmed these observations for magnocellular groups, with few apelinergic fibers, numerous OXY fibers, and little colocalization of apelin and OXY in fibers of the internal layer of the ME (Fig. 1, G–I).

TABLE 2. Mean number of neurons per section and per structure or subnucleus c-Fos-positive, c-Fos-positive/OXY-positive, and c-Fos-positive/OXY-negative in hypothalamic neuronal groups

	Number of c-Fos-positive neurons		Number of c-Fos-positive and OXY-positive neurons		Number of c-Fos-positive and OXY-negative neurons	
	aCSF	aCSF/K17F	aCSF	aCSF/K17F	aCSF	aCSF/K17F
Magnocellular groups						
Acc	2.5 ± 0.5	0.8 ± 0.3[a] $P < 0.02$	1.4 ± 0.3	0.3 ± 0.1[b] $P < 0.003$	1.0 ± 0.3	0.6 ± 0.2 n.s.
PaLM	10.0 ± 0.5	3.8 ± 0.6[c] $P < 1.8 \times 10^{-6}$	4.6 ± 0.3	1.1 ± 0.2[c] $P < 4.1 \times 10^{-8}$	5.5 ± 0.6	2.7 ± 0.5[b] $P < 0.002$
SO	23.6 ± 2.5	12.6 ± 1.1[c] $P < 0.001$	10.9 ± 1.2	4.1 ± 0.5[c] $P < 6.0 \times 10^{-5}$	12.7 ± 1.5	8.5 ± 1.0[a] $P < 0.03$
SOR	13.7 ± 1.0	4.5 ± 0.5[c] $P < 1.2 \times 10^{-6}$	6.0 ± 0.8	1.2 ± 0.1[c] $P < 3.2 \times 10^{-5}$	7.8 ± 0.6	3.3 ± 0.4[c] $P < 2.1 \times 10^{-5}$
Parvocellular groups						
PaAP	9.0 ± 1.3	5.0 ± 0.7[a] $P < 0.02$	3.1 ± 0.4	1.4 ± 0.3[b] $P < 0.005$	5.9 ± 1.1	3.7 ± 0.5 n.s.
PaDC	3.9 ± 0.4	3.8 ± 0.5 $P < 0.02$	1.6 ± 0.2	1.3 ± 0.2 $P < 0.005$	2.3 ± 0.3	2.5 ± 0.4 n.s.
PaMP	6.4 ± 0.6	3.6 ± 0.5[b] $P < 0.003$	2.7 ± 0.2	0.7 ± 0.1[c] $P < 1.1 \times 10^{-7}$	3.6 ± 0.5	2.8 ± 0.5 n.s.
PaPo	2.4 ± 0.5	1.2 ± 0.4 n.s.	1.2 ± 0.2	0.1 ± 0.1[b] $P < 0.004$	1.2 ± 0.4	1.0 ± 0.4 n.s.
PaV	6.3 ± 0.7	5.7 ± 0.5 $P < 0.003$	2.5 ± 0.2	1.8 ± 0.3[c] $P < 1.1 \times 10^{-7}$	3.8 ± 0.5	3.8 ± 0.4 n.s.
Magnocellular and parvocellular group						
Pe	4.8 ± 0.6	4.6 ± 0.5 n.s.	1.9 ± 0.1	1.7 ± 0.2 n.s.	2.9 ± 0.6	3.0 ± 0.5 n.s.

n.s., Not significant.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$.

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Apelin receptor mRNA expression in SON and PVN OXY neurons

Control sections incubated with the sense cRNA probe gave no signal (Fig. 2F). The double-labeling procedure, combining *in situ* hybridization for apelin receptor mRNA and immunohistochemistry for OXY, revealed that most magnocellular neurons, including those of the SON (Fig. 2, A and B), expressed apelin receptor mRNA. In the SON magnocellular neurons, the density of black grains in OXY-positive cells (3.2 ± 0.3 black grains/25 μm^2) was increased by 114% in comparison with areas not containing OXY-positive neurons (1.5 ± 0.2 black grains/25 μm^2); this difference was significant ($P < 0.0006$). In the anterior parvocellular part of the PVN (PaAP), which contains parvocellular neurons (Fig. 2, C and D), the density of black grains in OXY-positive cells (2.7 ± 0.2 black grains/25 μm^2) was increased by 54% in comparison with in areas not containing OXY-positive neurons (1.8 ± 0.2 black grains/25 μm^2); this increase was significant ($P < 0.0017$).

Distribution of apelin and TH immunoreactivities in the NTS/A2 region

We focused on the NTS caudal to the opening of the fourth ventricle (bregma, −13.30 mm) (35), which contains the noradrenergic A2 neurons projecting to magnocellular OXY neurons. The A2 region contained TH-positive neurons that were not immunolabeled for apelin (Fig. 3, A, C, D, and F). However, numerous apelin-positive fibers were observed among the noradrenergic cell bodies (Fig. 3, B, C, E, and F).

Activity of OXY hypothalamic neurons after the icv injection of K17F in lactating rats

Dual detection of c-Fos and OXY

In rats receiving icv injections of aCSF alone, the groups of neurons containing the largest number of neurons double-labeled for c-Fos and OXY were in the SON and magnocellular division of the PVN [the lateral magnocellular part of the PVN (PaLM)] (Table 2).

Intracerebroventricular injection of K17F decreased the number of c-Fos-positive and OXY-positive neurons in all the groups of purely magnocellular neurons (Table 2): the retrochiasmatic part of the SON (SOR) (≈80%) and the more rostral part of the SON (SO) (≈62%) (Fig. 4, A–C), the PaLM (≈76%) (Fig. 4, D–F) and Acc (≈78%). A similar decrease in the number of c-Fos-positive and OXY-positive neurons was observed in some parvocellular groups: the PaAP (≈55%) (Fig. 4, G–I), the medial parvocellular part of the PVN (PaMP) (≈74%) and posterior part of the PVN (PaPo) (≈92%). By contrast, icv injection of K17F did not influence other parvocellular groups, the dorsal cap of the PVN (PaDC) and the ventral part of the PVN (PaV), or the Pe, which contains both magnocellular and parvocellular neurons.

An analysis of the effect of icv K17F injection on the number of c-Fos-positive and OXY-negative neurons showed a decrease in OXY-negative neuron activity in all groups of purely magnocellular neurons other than in the Acc. In contrast, in parvocellular groups of neurons, OXY-negative neurons were not influenced by K17F.

Electrical activity of SON OXY neurons

The mean basal activity of the eight recorded OXY neurons was 4.7 ± 0.8 spikes/sec (Fig. 5C). The icv administration of K17F induced the gradual and sustained inhibition (up to 60 min after administration) of electrical activity in all neurons tested (Fig. 5, B and C): activity halved 20 min after the icv administration of K17F and continued to decrease thereafter (~95% decrease after 1 h). For the two cells displaying bursts during the control period, no burst was recorded after K17F in-

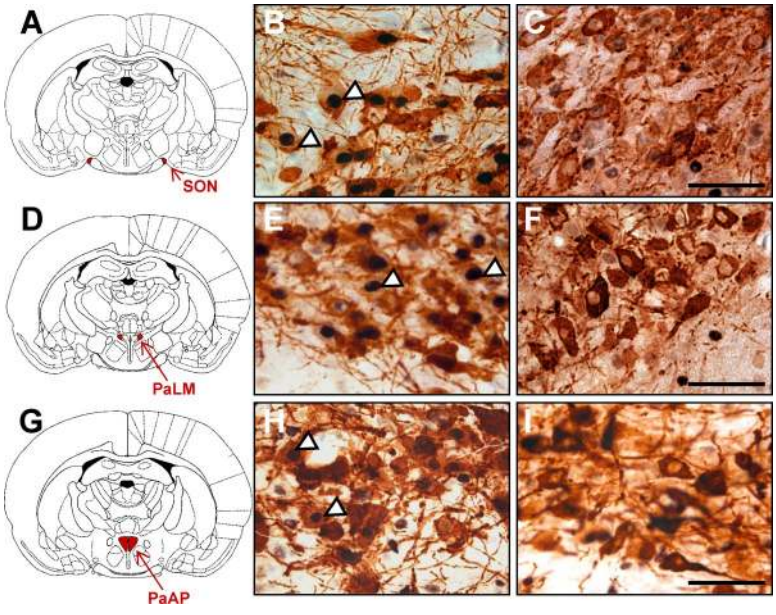

FIG. 4. Effect of apelin icv administered on *c-fos* expression in hypothalamic OXY neurons in lactating rats. Photomicrographs of coronal sections double-immunolabeled for c-Fos (*black*) and OXY (*brown*) in lactating rats receiving icv injections of K17F (4.7 nmol/rat; n = 9) (C, F, and I) or vehicle (aCSF; n = 8) (B, E, and H). Plates B and C are high magnifications of the SON at the level indicated in A (bregma, −1.80); plates E and F are high magnifications of the PaLM at the level indicated in B (bregma, −1.88); and plates H and I are high magnifications of the PaAP at the level indicated in C (bregma, −1.60). In magnocellular OXY neurons of the SON and PaLM and parvocellular OXY neurons of the PaAP, the icv administration of K17F decreased the number of double-labeled neurons (indicated by *arrowheads*). *Scale bar*, 50 μm.

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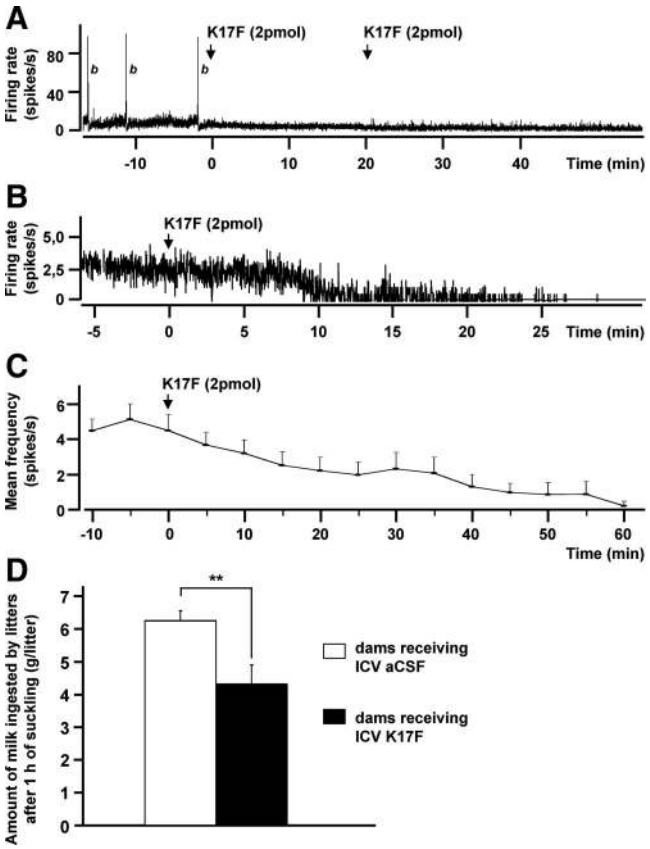

FIG. 5. Effect of apelin icv administered on the milk ejection reflex. The *traces* in A and B represent the evolution of the mean frequency (in spikes per second, bin width, 1 sec) of individual OXY magnocellular neurons before and after apelin injection. A, Typical example illustrating the inhibitory effect of apelin K17F (2 pmol) on a bursting OXY neuron during suckling. Apelin decreased basal activity and prevented the occurrence of bursts (b) during the 60-min period. B, Example illustrating the effect of apelin on a nonbursting OXY neuron during suckling. This neuron (identified as an OXY neuron on the basis of its excitatory response to cholecystokinin) (data not shown) displayed continuous basal activity without bursts and was strongly inhibited by apelin. It remained silent for more than 1 h after K17F injection (data not shown on the *trace*); during this silent period, stimulation of the neural stalk nonetheless induced an antidromic action potential with the same latency and response threshold as before the icv application of apelin, indicating an absence of loss of the recorded neuron. The graph in C shows the mean frequency of discharge (mean ± SEM in spikes per second, bin width, 5 min) of the eight OXY magnocellular neurons recorded in the SON of anesthetized rats after suckling. This trace clearly illustrated the installation and persistence of the inhibitory effect of K17F icv injected into the third ventricle. D, Effect of apelin on the amount of milk released to the pups. The amount of milk ingested by litters during 1 h of suckling dams receiving icv injections of K17F (4.7 nmol/rat; n = 9) or vehicle (aCSF; n = 8). Data are expressed as means ± SEM; **, $P < 0.01$. All OXY neurons displayed were recorded for at least 1 h after apelin injection, *i.e.* for a time corresponding to the suckling period allowed, in nonanesthetized dams (60 min). Both milk ejection and the amount of milk ejected depend on both the basal and bursting activity of OXY neurons. Thus, the inhibitory effect of apelin on electrical activity for more than 1 h is consistent with the significant decrease in the amount of milk obtained by the litter during 1 h of suckling (D).

jection, with a decrease in activity from basal levels being observed instead (Fig. 5A).

Amount of milk ingested by the litter after the icv injection of K17F

The weight of the litters after 6 h of separation did not differ between the group of mothers receiving icv injections of aCSF alone (142 ± 2 g) and those receiving icv injections of aCSF plus K17F (145 ± 2 g; $P < 0.2$).

After the return of the litters, the time taken for mothers to approach and sniff the pups, to retrieve all the pups, and to position themselves over the pups with legs splayed to accommodate the pups was similar for lactating rats receiving aCSF alone (7.0 ± 0.7 min) and for those receiving K17F (7.3 ± 0.7 min; $P < 0.4$).

Less milk was ingested (−31%; $P < 0.01$) (Fig. 5D) by the litters of dams receiving icv K17F injections (3.3 ± 0.5% increase in litter mass during 1 h of suckling) than by litters from dams receiving aCSF alone (5.0 ± 0.2% increase in litter mass during 1 h of suckling).

Discussion

The present study demonstrates that apelin interacts with OXY neurons in the hypothalamus in lactating rats (Fig. 6). By acting on apelin receptors expressed by magnocellular and parvocellular OXY neurons, apelin inhibits the activity of these neurons in an autocrine or paracrine manner, thereby decreasing the amount of milk ejected.

By combining *in situ* hybridization with immunohistochemistry, we showed that most of the PVN and SON magnocellular and parvocellular neurons immunoreactive for OXY contained apelin receptor mRNA in addition to OXY receptors (43), suggesting a possible role for these two neuropeptides in the regulation of OXY neurons.

In line with these data, we detected apelin-positive neuronal cell bodies in the magnocellular and parvocellular parts of the PVN and in the SON in lactating rats, as previously reported for male and for virgin and lactating female rats (8, 11, 12, 18, 22). We showed by double immunofluorescence labeling that less than 21% of magnocellular and parvocellular OXY neurons also contained apelin. These data are consistent with those reported by Brailoiu *et al.* (22) for Sprague Dawley rats, showing that magnocellular neurons immunoreactive for neurophysin I in the PVN and SON were immunoreactive for apelin. However, our results for the density of double-labeled neuronal cells differed markedly from those of this previous study, which reported that almost all the OXY neurons contained apelin. The use of Wistar rats, which have lower hypothalamic apelin levels than Sprague Dawley

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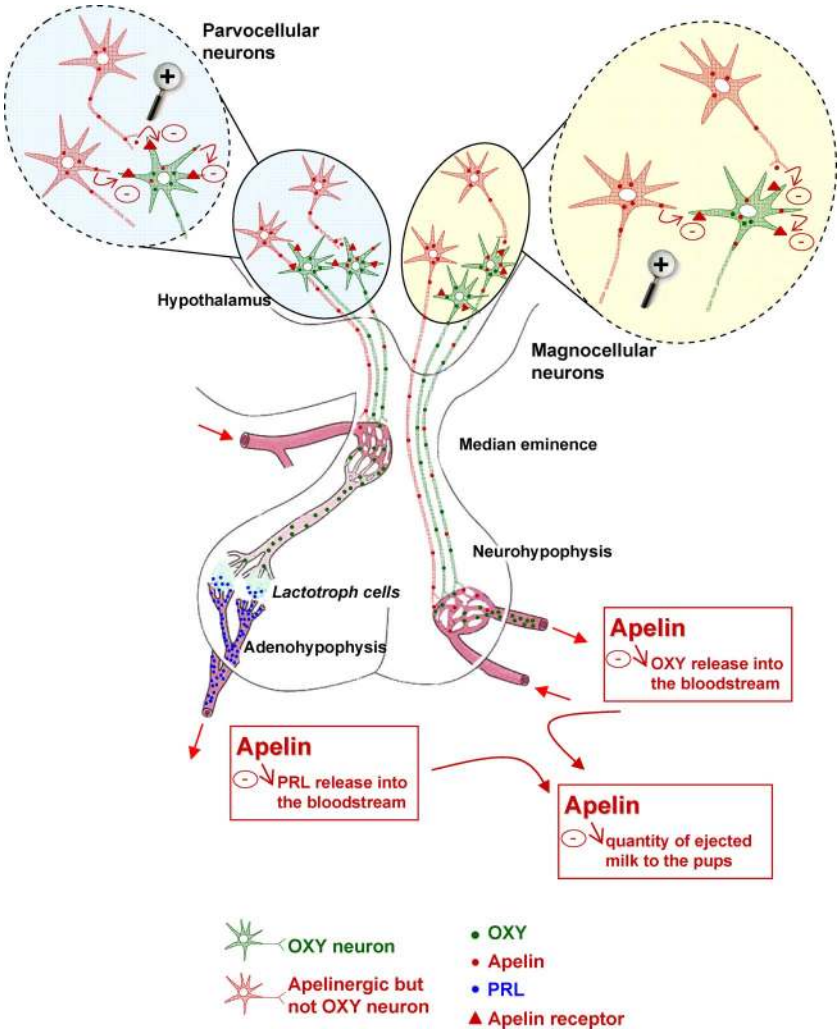

FIG. 6. Putative schematic diagram of the interactions between apelin and OXY hypothalamic neurons in lactating rats. Apelin, which exerts a direct inhibitory effect on hypothalamic OXY neuron activity, may be released from various neuronal populations: 1) hypothalamic OXY neurons also containing apelin and 2) neurons that are apelinergic but do not contain OXY. Thus, apelin may control hypothalamic OXY neuron activity in a direct autocrine or paracrine manner. The inhibitory control of magnocellular OXY neurons would decrease the amount of OXY released into the bloodstream, whereas this control on parvocellular OXY neurons may decrease the release of PRL into the bloodstream by decreasing the stimulatory effect of OXY on lactotrophs in the adenohypophysis. Both effects would decrease the amount of milk ejected for the pups. The magnifying glass with a *plus symbol* indicates that the area bounded by a *dotted line* corresponds to an enlargement of the area bounded by a *solid line*, in parvocellular neuronal population on the *left* and in magnocellular neuronal population on the *right*.

rats (8), and in the choice of the physiological state (lactating), may account for this difference. Overall, these data suggest that apelin directly regulates PVN and SON magnocellular and parvocellular OXY neurons in a paracrine and autocrine fashion, via the apelin receptors expressed by OXY neurons. Paracrine regulation of OXY neuron activity by apelin may occur not only through the release of apelin from SON and PVN OXY/apelin neurons but also through the release of apelin from SON and PVN AVP neurons, in which apelin has been shown to colocalize strongly with AVP (Fig. 6) (8, 12). It is also possible that endogenous apelin regulates the activity of A2 noradrenergic neurons in the NTS known to project to the PVN and SON, controlling magnocellular OXY neuron activity, and participating in the milk ejection reflex (25, 27, 28, 44). However, we showed that apelin did not colocalize with TH in A2 noradrenergic neurons in the NTS, being instead found in apelinergic fibers within this nucleus, as already reported (18), among the noradrenergic cell bodies.

Apelin and its receptors are strongly expressed in the hypothalamus, by OXY neurons, but also by other neuronal populations. We therefore investigated the effect of apelin on the activity of OXY neurons in lactating rats, by measuring *c-fos* expression in these neurons. We observed that the infusion of K17F into the lateral ventricle inhibited the neuronal activity of most of the hypothalamic OXY neurons (*i.e.* magnocellular and parvocellular neurons). We also measured, in anesthetized lactating rats, the effect of apelin on the electrical activity of the magnocellular OXY neurons of the SON displaying a strong rhythmic pattern during lactation. The infusion of K17F into the third ventricle inhibited the basal and bursting activity of these neurons, and this would be expected to decrease OXY release from neurohypophyseal axon terminals. These data suggest that apelin released from OXY or AVP neurons during breastfeeding directly inhibits magnocellular and parvocellular OXY neurons in an autocrine or paracrine manner, via the apelin receptors expressed on these neurons.

Magnocellular OXY neurons are activated by the suckling pups, leading to the release of OXY into the bloodstream, where this compound acts on the myoepithelial cells of the mammary gland, causing smooth muscle contraction and milk ejection (25). We therefore quantified milk delivery to the pups after the icv injection of K17F in conscious lactating rats. We observed a marked decrease in milk ejection after K17F injection, suggesting that brain apelin decreases the supply of nutrients from the mother to the litter (Fig. 6). The inhibitory effect of apelin on magnocellular OXY neurons is not consistent with the findings of a recent study reporting an absence of effect of apelin 13

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on the firing rate of these neurons (15). This difference may be due to the physiological status of the animals (lactating rats here *vs.* virgin rats in the other study) and to the mode of administration of apelin (icv route here *vs.* diffusion over the ventral surface of the SON through a dialysis probe). Magnocellular OXY neurons have been shown to have higher rates of activity in lactating rats than in virgin rates, with bursts of activity consisting of 1–2 sec of intense discharge occurring at intervals of 1–15 min (45). The apparent discrepancy between our results and those of the previous study (15) may therefore be due to differences in the hormonal status of the animals. Such a difference in the effect of apelin on neuronal activity between animal models has also been reported for AVP neurons: apelin inhibits the firing of these neurons in lactating rats (8) but increases their activity in virgin rats (15). The inhibitory effect of apelin on OXY magnocellular neurons may play a determinant role in controlling the basal level of activity of these neurons and, consequently, their ability to display a burst of activity in response to suckling. Indeed, as suckling proceeds, the expression of a burst of activity is known to be strongly linked to the mean level of basal activity for the OXY magnocellular population with a critical firing rate (between one and three spikes per second) (46). Central administration of OXY has been shown to increase the basal activity of OXY neurons with initially low levels of basal activity (less than three spikes per second) (47) and to decrease the activity of OXY neurons initially firing at high levels when bursts of activity are induced at the start of suckling (46). Consequently, during the milk ejection reflex, apelin may play a modulatory role, antagonistic to that of OXY, and resulting in adjustment of the basal activity of the whole OXY neuron population toward the critical level required for a burst of activity.

Parvocellular OXY neurons projecting to the external layer of the ME, such as those of the PaAP or PaMP, which displayed a decrease in *c-fos* expression after the icv injection of K17F, are thought to release OXY into the hypophyseal portal circulation to increase directly the release of adenohypophysis PRL from lactotrophs known to express OXY receptors (29–31, 48). Thus, the inhibitory effect of apelin on this subpopulation of OXY neurons may decrease the release of PRL into the bloodstream, thereby also decreasing milk production. The smaller amount of milk ejected after icv injection of K17F may therefore also be linked to lower levels of milk production due to the inhibition of parvocellular OXY neurons projecting to the external layer of the ME (Fig. 6). Consistent with this hypothesis, a decrease in plasma PRL levels was previously observed after the icv administration of apelin (49).

In conclusion, in lactating rats, we have demonstrated the coexistence of apelin receptor mRNA and, to a lesser extent, apelin, with OXY in magnocellular and parvocellular OXY neurons of the PVN and SON and the inhibitory effect of apelin on OXY neuron activity, markedly decreasing the amount of milk delivered to the pups. These findings provide evidence for the autocrine and paracrine inhibitory regulation of OXY neurons by apelin, through the modulation of bursts of activity in OXY magnocellular neurons and control over the activity of parvocellular OXY neurons. Together with the crucial role played by apelin in the PVN and SON in the maintenance of body fluid homeostasis through the regulation of AVP neuron activity, our data suggest that apelin in these nuclei plays a key role in the regulation/control of OXY neuron activity during breastfeeding. Thus, apelin, which limits the action of OXY during lactation and counteracts the antidiuretic action of AVP, may be an important peptide for the fine regulation of functions relating to the maintenance of body fluid homeostasis in certain physiological conditions.

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