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**Oxytocinase in the female rat hypothalamus: a novel mechanism controlling oxytocin neurons during lactation**

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**Abbreviated title:** Hypothalamic oxytocinase regulates oxytocin neurons.

## Abstract

In addition to its peripheral actions, oxytocin released within the brain is important for birth and essential for milk ejection. The oxytocinase enzyme (placental leucine aminopeptidase, P-LAP) is expressed both peripherally and centrally. P-LAP controls oxytocin degradation in the uterus, placenta and plasma during pregnancy, but its role in the hypothalamus is unclear. We investigated P-LAP expression and activity in the hypothalamus in virgin, pregnant and lactating rats, and its role *in vivo* during the milk-ejection reflex. P-LAP mRNA and protein were expressed in magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei. Oxytocin neurons co-expressed P-LAP without strong subcellular co-localisation of oxytocin and P-LAP, indicating that they are packaged in separate vesicles. Examination of the intracellular distribution of oxytocin and P-LAP showed a redistribution of P-LAP to within 1  $\mu\text{m}$  of the plasma membrane in the somata of oxytocin neurons during lactation. Both P-LAP mRNA expression and hypothalamic leucyl/cystinyl aminopeptidase activity in the soluble fraction were higher during lactation than in late pregnant or virgin states. Inhibition of central enzyme activity by i.c.v. injection of amastatin in anaesthetized suckling mothers increased the frequency of reflex milk ejections. As hypothalamic P-LAP expression and activity increase in lactation, and prevention of its action mimics central oxytocin administration, we conclude that P-LAP regulates auto-excitatory oxytocin actions during the suckling-induced milk-ejection reflex.

## Introduction

Oxytocin is synthesized in the somata of magnocellular neurons in four hypothalamic nuclei; the paired supraoptic (SON) and paraventricular nuclei (PVN). It is packaged in large dense-cored vesicles which are transported to axon terminals in the neurohypophysis: from these terminals, oxytocin is secreted into the general circulation. In the rodent, during both parturition and the milk-ejection reflex, the magnocellular oxytocin neurons exhibit intermittent, quasi-synchronous bursts of electrical activity, typically every 5-10 min, which trigger pulsatile oxytocin secretion from the terminals. This pulsatile mode of oxytocin secretion is important for the progress of parturition and is essential for milk transfer to the young during lactation (1-4), and it critically depends on release of oxytocin from the somata and dendrites of oxytocin neurons (5). Increased somato-dendritic release of oxytocin precedes, and is required for, the co-ordination of bursting activity: the frequency of these bursts can be accelerated by i.c.v. injection of oxytocin or delayed by i.c.v. injection of an oxytocin antagonist (1, 6, 7).

The availability of hormones and transmitters is regulated not only by the amount released but also by the rate of degradation. In the periphery, serum levels and uterine and placental expression of the oxytocin degrading enzyme, placental leucine aminopeptidase (P-LAP), increase during mid- to late pregnancy (8, 9). This increased expression of P-LAP is thought to be important for preventing premature uterine contractions, thereby helping to maintain normal pregnancy to term (10, 11), and deletion of P-LAP in a transgenic mouse model advances the onset of birth (12). However, P-LAP also degrades vasopressin, angiotensin III, met-enkephalin and dynorphin (13), and central injections of the P-LAP inhibitor amastatin have been shown to modify drinking behavior (14, 15).

In peripheral tissues, the enzyme is found in the same intracellular vesicles as the glutamate transporter GLUT4, and is activated upon translocation from the cytosol to the

plasma membrane (16-18). Inserted into the plasma membrane, P-LAP is a membrane-spanning protein (19), which is active in the extracellular domain. In addition, the enzyme can be freed from the plasma membrane into the extracellular space by sheddases (20). Although P-LAP is thought to be active physiologically only after insertion into the plasma membrane, P-LAP activity is measurable in both membrane-bound and cell associated-soluble fractions of tissue homogenates (21).

P-LAP was recently identified in the hypothalamus (22) and inhibition of P-LAP activity with amastatin has been shown to mimic the effect of exogenous oxytocin administration on SON neuronal activity in a hypothalamic slice preparation (23). There have been two conflicting reports of the effect of knocking out the P-LAP gene on parturition, one without effect (24), the other showing a significant shortening of pregnancy duration (12). In this latter study, wild-type mice given continuous subcutaneous infusion of recombinant P-LAP showed a delay in the onset of labour.

However, there have been no studies to determine if central P-LAP plays a role in the control of oxytocin-dependent activity *in vivo*. In this study, we first visualized P-LAP mRNA and protein expression in the hypothalamus, and investigated its intracellular distribution and co-localization with oxytocin-neurophysin using immunofluorescence. Pregnancy- and lactation-associated changes in hypothalamic P-LAP mRNA expression were analyzed by *in situ* hybridization, and changes in cystinyl/leucyl aminopeptidase activity in both membrane-bound and soluble fractions from samples of hypothalamus and posterior pituitary were assayed *in vitro*. Finally, we investigated the effect of inhibiting central P-LAP enzyme activity *in vivo* on the milk-ejection reflex.

## Methods

### *Experimental Animals:*

Adult female Sprague-Dawley rats (initial body weight 200-280g, Bantin & Kingman, UK) were either used as virgins, on day 19-21 of pregnancy or during lactation (post-natal day 7-11, day of birth: being post-natal day 1). The day of finding a vaginal plug in females caged singly with a male was taken as day 1 of pregnancy. Rats had *ad libitum* access to food and water and 12 h of light per day (lights on: 07:00h) and ambient temperature was on average 20-21°C. All procedures were carried out in accordance with UK Home Office Animals (Scientific Procedures) Act, 1986 legislation and were approved by the University of Edinburgh Ethical Committee.

### *Collection and processing of brain tissue.*

Rats were anaesthetized with an overdose of sodium pentobarbitone (120mg/100g body weight, i.p.) and perfused transcardially with heparinised (5000 U/ml; 300 ml) physiological saline (0.9%) followed by paraformaldehyde (4%) in 0.1M phosphate buffer (PB; pH 7.2). Brains were removed and post-fixed overnight in 2% paraformaldehyde and 15% sucrose in 0.1M PB at 4°C. The tissues were cryoprotected in 30% sucrose in 0.1M PB (until brains had fully sunk, usually 48h). The brains were snap-frozen on dry ice then sectioned coronally at 40 µm using a freezing microtome.

### *Colocalization of P-LAP with oxytocin-neurophysin or GM130:*

Sections from virgin, day 22 pregnant and lactating rats (4 sections containing SON per rat, 4 rats per group) were labelled for oxytocin-neurophysin and either P-LAP or the Golgi marker GM130 and visualized with fluorescent probes to analyze their subcellular co-localization

and distribution. In brief, sections were incubated in P-LAP antibody (gift from Dr Tsujimoto, Riken Brain Institute; Japan; rabbit polyclonal; 1:1000) and either an oxytocin-neurophysin antibody (PS38, a gift from Professor Hal Gainer, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, USA; mouse monoclonal; 1:5000) or GM130 antibody (BD Transduction Laboratories; mouse monoclonal; 1:1000), for 48h. This was followed by incubation with a biotinylated anti-rabbit secondary antibody (Vector Laboratories, UK, 1:500 for 2h at room temperature in the dark), then streptavidin conjugated-Alexa Fluor 488 (green, to visualize P-LAP) and Alexa Fluor 568 anti-mouse secondary antibody (red, to visualize oxytocin-neurophysin or GM130; both Life Technologies, UK, each at 1:500). After further washing, the sections were mounted using a Moviol-based medium (Calbiochem, San Diego, CA, USA), supplemented with 2.5% DABCO (1, 4-diazabicyclo[2.2.2]octane, Sigma).

Fluorescence signals were acquired using a Zeiss LSM510 Axiovert Confocal laser scanning microscope, equipped with argon/krypton lasers. Emissions from each fluorescently-labelled secondary antibody were acquired consecutively to ensure no bleed between channels. Signals were acquired at 1024x1024 pixels, using a Zeiss Plan NeoFLUAR 1.4 NA x63 oil-immersion objective. For intracellular co-localisation analysis, images of SON oxytocin neurons were taken throughout each cell at Nyquist sampling rates which were deconvolved using Huygens software (Scientific Volume Imaging). For each cell, the middle layer was identified and the image cropped to the outline of the cell, and the resultant image was examined using NIH Image J software to calculate the degree of co-localization (quantified by Pearson's co-efficient of variation). This was repeated for 15 cells each from virgin female, D22 pregnant and D8-11 lactating rats and data compared. Cells in each treatment group were averaged and compared using a one-way analysis of variation followed by Tukey's *post-hoc* test. In addition, the distributions of both the oxytocin-

neurophysin and P-LAP signals from the cell nucleus to plasma membrane were investigated in SON oxytocin neurons using ImageJ software. The mid-optical layer of each cell was separated into two layers, one for each channel (red and green). For each cell, 16 lines were drawn from the cell nucleus to the plasma membrane and measurements of signal intensity of both channels made with respect to the line distance. For each signal, the intensity of signal was expressed as a percentage of the total signal intensity for the length of the line. The measurements were binned into 1 $\mu$ m segments so that the normalised proportion of signal for oxytocin-neurophysin and P-LAP in the 1 $\mu$ m closest to the nucleus and 1 $\mu$ m closest to the plasma membrane for each cell could be averaged. This was repeated for 15 cells each from virgin female, D22 pregnant and D8-11 lactating rats and data compared.

No fluorescent labelling was detected when primary antibodies were omitted. P-LAP (25) and oxytocin-neurophysin antibody specificity have been described previously (26, 27) and the GM130 primary antibody specificity is described on BD Transduction Laboratories website (catalogue number 610822).

### ***P-LAP mRNA expression***

P-LAP mRNA expression was analyzed by quantitative *in situ* hybridization in virgins (n=6), and rats on day 20-21 of pregnancy (n= 6) or day 8-9 of lactation (n=7). The litter size ranged from 11-18 pups (median=13) in the pregnant group and 11-15 pups (median=12) in the lactating group. All lactating mothers exhibited maternal behaviors including licking/grooming and suckling pups, and milk could be seen in the stomachs of the pups. The rats were killed between 10:00-12:00h and their brains removed and frozen immediately on dry ice, as above. Placentae were also collected from the pregnant rats for use as positive control and negative controls.

*Preparation of P-LAP probe:* A rat P-LAP cDNA (28) was generously provided by Dr Tsujimoto (Riken Brain Institute, Japan). <sup>35</sup>S-UTP labelled cRNA sense and antisense probes were synthesized from the linearized pCI-neo vector expressing a 3075 base-pair cDNA fragment encoding rat P-LAP. The plasmid was linearized with MluI and EcoRI and transcribed using T7 and T3 polymerases (Promega UK Ltd., Southampton, UK), for the sense and antisense riboprobes, respectively. To aid tissue penetration, the radiolabelled P-LAP riboprobe was fragmented by alkaline hydrolysis (60°C for 20 min) and neutralized before hybridization.

*In situ hybridization :* Whole brains were cryostat-sectioned coronally at 15µm and thaw mounted onto DNase/RNase free Poly-lysine® glass microscope slides (VWR) and stored at -70°C. Slides were selected from the SON and PVN for each rat and were processed by *in situ* hybridization as previously described (29). Coronal sections of placenta treated as above were used as positive controls (see Fig 4A left). Brain and placenta sections serving as negative controls to ensure probe specificity were hybridized with <sup>35</sup>S-UTP labelled cRNA sense probes (see Fig 4A right for placenta negative control). After overnight hybridization, the slides were rinsed briefly in 2x saline sodium citrate (SSC) and then washed 3 x 5 min in 2x SSC at room temperature. Next, sections were incubated in buffer (1mM EDTA, 500mM sodium chloride, 10mM Tris, pH 7.6) containing 15µg/ml RNase A for 60 min at 37°C. Sections were then briefly rinsed in 2X SSC at room temperature before further stringency washing (60 min each in 2x SSC at 50°C, 0.2x SSC at 55°C, then 0.2x SSC at 60°C). Following the post-hybridization washes tissue was dehydrated in an ascending series of ethanol containing 300 mM ammonium acetate and air-dried. Slides were then either exposed

to autoradiographic film for 13 days or dipped in emulsion (K-5, Ilford, Knutsford, Cheshire, UK), and incubated at 4°C for 5 weeks.

*Quantification:* After developing, fixing (Kodak D-19, Sigma; Hypam rapid fixer, Ilford) and counterstaining with haematoxylin and eosin, autoradiographs were quantified in emulsion-dipped slides. The silver grain area overlying each magnocellular SON and PVN region and the area of each nucleus profile was measured in 9 consecutive sections per rat (NIH Image software, v1.62) and grain density calculated as grain area divided by nucleus profile area ( $\text{mm}^2/\text{mm}^2$ ). Background measurements were made over areas adjacent to the region of interest and subtracted. The hybridization signal over tissue hybridized with the sense probe was not different from background.

### ***Aminopeptidase activity***

Both soluble and membrane-bound aminopeptidase activities were assayed for virgin (n=7), 19-21 day pregnant (n=7) and 7-8 day lactating (n=6) rats. The rats were deeply anaesthetized with pentobarbitone (120mg/100g body weight, i.p) and transcardially perfused with ice-cold isotonic saline (4°C) for 5-10 min (~100ml) before their tissues were collected and frozen on dry ice. A thick coronal slice containing the hypothalamus was cut from the brain and the hypothalamus (including the SON and PVN, ~50mg), the pituitary was removed and the neurohypophysis (posterior pituitary) separated from the pars intermedia and adenohypophysis; the cerebral cortex was collected as a control for regional specificity. Samples of placenta and pregnant uterus were also collected for analysis as positive control tissues (20% of one placenta and ~50mg uterus containing an implantation site were collected from each pregnant rat).

Both leucyl aminopeptidase (Leu-AP) and cystinyl aminopeptidase (Cys-AP) activities were measured as independent and complementary indicators of oxytocinase activity. Activity was assayed as amount of substrate hydrolyzed by soluble or membrane-bound fractions. To generate the two fractions, tissue samples were homogenized in ice-cold 1ml Tris/Cl buffer (pH 7.4) for 1 min at 900 rpm and then ultra-centrifuged at 100,000g for 30 min at 4°C. The supernatant was collected and kept overnight at 4°C until assay for soluble enzyme activity. The pellet was then re-homogenized in 1ml TrisCl buffer containing 1% Triton X-100 as before and centrifuged again at 100,000g for 30min at 4°C. The supernatant was incubated with Biobeads (polymeric adsorbant; Biorad) for 2h at 4°C on a rotating shaker to capture the detergent which would inhibit aminopeptidase activity. The supernatant was collected and stored at 4°C overnight until assay for membrane-bound enzyme activity. Aliquots were analyzed for Leu-AP- and Cys-AP activity and for protein content. For enzyme activity, samples (and control blanks) were incubated with shaking for 30min at 37°C with 1ml substrate solution (either 2.92mg/100ml leucyl- $\beta$ -naphthylamide in 50mM Tris/Cl buffer pH 6.0 or 5.63mg/100ml cystinyl- $\beta$ -naphthylamide in 50mM phosphate buffer pH 7.4; both with 10% dithiothreitol and 10% fraction V bovine serum albumin, BSA). The reaction was stopped with 1ml 0.1M acetate buffer pH 4.2 on ice. Low (range 2000-7000 pmole) and high (range 7000-50,000 pmole) standard curves (depending upon enzyme activity level), were generated by incubating increasing concentrations of 2-naphthylamine in TrisCl buffer containing dithiothreitol and BSA as above and stopped with acetate buffer on ice. Fluorescence was read at 345nm excitation and 412 nm emission on a spectro-fluorimeter, and enzyme activity calculated by reading from the curve. Protein in the samples was estimated by the Bradford assay, reading the absorbance at 595nm on a spectrophotometer. Activity was expressed as nM substrate (Leu- or Cys-naphthylamide, NA) degraded/min/mg total protein.

### ***The milk ejection reflex***

The effects of i.c.v. oxytocin, which increases the frequency of the milk ejection reflex (30), with i.c.v. amastatin, an oxytocinase-inhibitor were compared (31, 32). Lactating (post-natal day 8-10, n=5) rats with at least 6 pups were separated from all but one pup overnight, and the following morning were anaesthetized with urethane (ethyl carbamate 1.1g/kg body weight, i.p.). A cannula filled with heparinized 0.9% saline was placed in the left femoral vein for injection of standard oxytocin solutions to calibrate the mammary gland responses. Saline-filled cannulae were placed in at least one of the left nipple ducts to record intra-mammary pressure (*via* pressure transducers connected to a CED 1401 A/D interface connected to a computer). The intra-mammary pressure measurements were acquired and analyzed using Spike2 (v6, Spike 2 software; CED, Cambridge UK). For i.c.v. administration of drugs, a 21 gauge guide cannula was inserted into the left lateral cerebral ventricle (4.5 mm from top of the skull; 0.1mm caudal and 0.6mm lateral to bregma). 1 h after completion of surgery, the hungry pups were placed at the nipples and allowed to suckle; further experimental procedures continued only after at least 6 pups were attached to the nipples. The sensitivity of the mammary glands to the concentration of circulating oxytocin was assessed using i.v. injections of 0.1, 0.25 and 0.5mU of oxytocin in isotonic saline. These exogenous oxytocin “pulses” convert to 0.2 ng, 0.5 ng and 1.0 ng respectively. These injections were repeated at the end of the experiment to verify the oxytocin-sensitivity of the mammary glands was unchanged and to provide dose-response curves. The dose-response curves were used to convert the amplitude measurements of the sharp increases in intra-mammary pressure to plasma concentrations of endogenous oxytocin pulses. At least 30 min after checking mammary gland sensitivity and recording a control period of milk ejection reflexes, an i.c.v. injection was made of either oxytocin (1  $\mu$ mol in 2  $\mu$ l artificial cerebrospinal fluid,

aCSF) or amastatin (6 nmol in 2  $\mu$ l aCSF). The milk-ejection frequency in the 15 min following treatment was compared to that in the 15 min pre-injection period. A second i.c.v. injection was not made until at least 30 min after the milk-ejection frequency returned to the control frequency. I.c.v cannula placement was verified post mortem after injection of dye (2  $\mu$ l 1% alcian blue dye) into the cerebral ventricles.

## Statistics

Data were analyzed using SigmaStat software. Kruskal-Wallis one-way Analysis of Variance (ANOVA) on ranks followed by Tukey's *post-hoc* test was used to compare expression, co-localization and activity analysis across the groups. Characteristics of the milk ejection reflex (frequency and amplitude) were compared using unpaired t-tests.  $P < 0.05$  was considered significant. The data shown are means  $\pm$  S.E.M.

## Results

### *P-LAP expression*

Immunocytochemistry revealed labelling of P-LAP in SON and PVN neurons, including both magnocellular and parvocellular (PVN) neurons (Fig. 1A-D). In SON and PVN magnocellular neurons, P-LAP immunoreactivity was observed in both the somata and the dendrites (Figs 1A, D and 2A [lactating], arrowheads) of labelled oxytocin neurons. P-LAP labelling was also observed in oxytocin-negative neurons (Fig 1D, arrows), presumed (in the SON) to be magnocellular vasopressin neurons. Other P-LAP labelled regions included the ventromedial hypothalamic nucleus, arcuate nucleus, suprachiasmatic nucleus, cortex and

hippocampus (not shown), consistent with the previously reported distribution of P-LAP in the rat brain (20).

*Analysis of cellular co-localization with oxytocin-neurophysin or a Golgi marker*

Images of SON that were taken through deconvolution processing showed punctate P-LAP immunoreactivity in both oxytocin neurons and in non-oxytocin (presumptively vasopressin) neurons (Fig. 2). SON neurons have a subplasmalemmal cortical layer of actin (33), and labelling F-actin with Alexa 647-phalloidin was used to define the boundaries of individual somata and proximal dendrites. Each image was cropped in X-, Y- and Z-projection so that just the middle layer of a single cell was shown. These cropped images were then used to analyse P-LAP and oxytocin-neurophysin co-localisation. In cells from control and pregnant rats there was overlap of the areas of the cell in which P-LAP and oxytocin-neurophysin were seen. Although a high Pearson's co-efficient of co-variance was measured (Fig 2C), it was not perfect indicating that P-LAP may be present in different vesicles from those that contain oxytocin but that the oxytocin and P-LAP containing vesicles travel in similar parts of the cell cytoplasm. There was a significant decrease in the co-variance of oxytocin and P-LAP in lactating rats compared to virgin rats (Pearson's coefficient of variation: virgins  $0.88 \pm 0.02$  pregnant  $0.79 \pm 0.03$ ; lactating  $0.66 \pm 0.05$ ;  $P < 0.05$  lactating vs virgins by Kruskal-Wallis one-way ANOVA on ranks;  $H = 13.686$ , Fig. 2C). This may be due to the greater rate of oxytocin secretion from the somato-dendritic compartment during lactation with perhaps the retention of P-LAP at the plasma membrane.

Additional images were used to determine the colocalisation of P-LAP and the Golgi marker GM130 in SON magnocellular neurons (not exclusively oxytocin cells; Fig. 2B,D). Strong P-LAP immunoreactivity was consistently seen in the perinuclear region where the Golgi apparatus is typically found. Analysis of colocalization of P-LAP with GM130 showed

no significant difference in average Pearson's coefficient of variation among control, pregnant and lactating rats (Fig. 2D).

Line analysis was used to examine the subcellular distribution of P-LAP and/or oxytocin-neurophysin within the soma cytoplasm, using the same mid-cell layer from individual oxytocin cells described above. Sixteen radiating lines were placed across the cell, starting at the nucleus and ending at the plasma membrane. The signal intensity in the pixels covered by the line and occupied by each channel in 1- $\mu$ m intervals (bins) was calculated for each line, and those in the first (perinuclear area) and last (plasma membrane) bins were averaged for the 16 lines on that cell (Figs 3). In cells from lactating rats, for both oxytocin-neurophysin and P-LAP, less of the immunoreactivity was found in the perinuclear area and more at the plasma membrane compared to cells from virgin controls (% oxytocin-neurophysin in perinuclear area: virgins  $23.0 \pm 2$ , pregnant  $16.6 \pm 1$ , lactating  $16.6 \pm 1.1$ ;  $P < 0.05$  vs virgins;  $H = 24.2$ ; %oxytocin-neurophysin near plasma membrane: virgins  $9.9 \pm 0.8$ , pregnant  $8.6 \pm 0.95$ , lactating  $15.5 \pm 1.2$ ;  $P < 0.05$  vs lactating;  $H = 61.3$ ; % P-LAP in perinuclear area: virgins  $25.11 \pm 1.8$ , pregnant  $20.2 \pm 1$ , lactating  $20.1 \pm 1$ ;  $P < 0.05$  vs lactating;  $H = 20.7$ ; %P-LAP near plasma membrane: virgins  $10.2 \pm 0.7$ ; pregnant  $8.8 \pm 0.8$ ; lactating  $14.3 \pm 0.9$ ;  $P < 0.05$  vs lactating,  $H = 52.9$ ; Fig. 3). Cells from pregnant rats showed an intermediate state between virgin and lactating (no significant difference to the lactating group in the perinuclear area, but less than in the virgin group for both oxytocin and P-LAP). Conversely, at the plasma membrane there was no significant difference compared to the virgin group, but significantly less of the oxytocin and P-LAP immunoreactivity was at the plasma membrane than in the lactating group. All of this suggests that during lactation when there is an increased demand on somato-dendritic secretion, there is a re-distribution of both oxytocin and P-LAP from the perinuclear area to the plasma membrane.

#### *Effect of pregnancy and lactation on P-LAP mRNA expression in the hypothalamus*

Strong P-LAP mRNA hybridization was observed in the SON and PVN (Fig. 4B, C), although expression was less dense than in the placenta (Fig. 4A, left anti-sense positive control). There was a complete lack of P-LAP mRNA hybridisation in the placental sense negative control (Fig 4A, right). As with the immunocytochemical labelling, hybridization was observed in other hypothalamic regions, including the ventromedial hypothalamus, arcuate nucleus and supra-chiasmatic nucleus (not shown). Some non-hypothalamic brain areas including the cortex also exhibited hybridization. In the magnocellular PVN, grain density was greater in lactating rats than in virgin or late-pregnant rats (both  $P < 0.05$ ,  $F = 5.841$ , d.f. = 16; Fig 4D). In the SON there were no significant differences in grain density with reproductive status (virgin:  $0.77 \pm 0.07$ ; pregnant:  $0.96 \pm 0.2$ ; lactating:  $0.63 \pm 0.08$  mm<sup>2</sup>/mm<sup>2</sup>; Fig. 4C).

#### *Effect of pregnancy and lactation on aminopeptidase activity*

Measurements of Cys-AP activity and Leu-AP activity showed a generally consistent pattern: lactating rats had higher mean levels of activity than virgins in both the posterior pituitary and hypothalamus, for both Cys-AP and Leu-AP activity, in the soluble fractions (posterior pituitary: Cys-AP,  $P < 0.05$ ,  $F = 7.19$ ; Leu-AP,  $P < 0.05$ ,  $F = 4.17$ ; hypothalamus: Cys-AP,  $P < 0.05$ ,  $F = 20.07$ ; Leu-AP,  $P < 0.05$ ,  $F = 8.1$ ; Fig. 5). The enzyme activity in membrane-bound fractions was also significantly higher in lactating rats for Cys-AP and Leu-AP activity in the posterior pituitary, but not hypothalamus (posterior pituitary: Cys-AP,  $P = 0.09$ ,  $F = 2.7$ ; Leu-AP,  $P < 0.05$ ,  $F = 13.49$ ; hypothalamus: Cys-AP,  $P < 0.05$ ,  $F = 4.31$ ; Leu-AP,  $P = 0.47$ ,  $F = 0.78$ ; Fig. 5). In hypothalamic fractions, the mean level of Leu-AP activity consistently exceeded that of CysAP activity, whereas the converse was the case for posterior pituitary fractions.

These differences with reproductive state were regionally specific. In the cerebral cortex, there were no significant differences between groups for Leu-AP or Cys-AP activity in the soluble fraction (Leu-AP: virgin  $19768 \pm 2123$ ; pregnant  $13103 \pm 2049$ ; lactating  $18639 \pm 4247$  NA/min/mg total protein; Cys-AP: virgin  $4400 \pm 330$ ; pregnant  $4224 \pm 549$ ; lactating  $5508 \pm 874$  NA/min/mg total protein). In the membrane-bound fraction, there was more Leu-AP activity in the cortex in pregnant rats ( $15214 \pm 1358$ ) than in virgin ( $4728 \pm 514$ ) or lactating rats ( $917 \pm 822$  NA/min/mg protein; both  $P < 0.05$ ,  $F = 40.14$ ) but no significant differences in the membrane-bound fraction (virgin  $2512 \pm 217$ ; pregnant  $3243 \pm 702$ ; lactating  $2516 \pm 490$  NA/min/mg protein).

Uterus: Similar to previous reports (8), Leu-AP activity in the soluble fraction of the uterus was similar between groups (data not shown), but there was more membrane-bound activity in pregnant rats than in virgin rats ( $28356 \pm 3408$  and  $8344 \pm 964$  NA/min/mg total protein, respectively, t-test  $P < 0.05$ ).

#### *Effect of blocking oxytocinase activity on the milk-ejection reflex*

Intramammary pressure recordings were made in anaesthetised suckling mothers. Central amastatin administration (6 nmole in  $2 \mu\text{l}$ ) rapidly increased the frequency of the milk-ejection reflex to a similar extent as oxytocin (1  $\mu\text{mole}$  in  $2 \mu\text{l}$  saline i.c.v., Fig. 6). The frequency of milk ejections significantly increased by  $224 \pm 15\%$  after i.c.v. oxytocin and by  $268 \pm 29\%$  after i.c.v. amastatin ( $P < 0.05$ , t-test,  $n = 5$ ). However, the average amplitude of intramammary pressure did not significantly change with either oxytocin or amastatin treatment. The intramammary pressure changes in response to known concentrations of intravenously applied oxytocin were used to calculate the amount of oxytocin released during each milk ejection reflex. Increasing central oxytocin either by icv injection of oxytocin or

amastain resulted in a significant increase in the amount of oxytocin released from the pituitary (from  $1.5 \pm 0.09$  to  $3.5 \pm 0.49$  and  $0.8 \pm 0.04$  to  $3.1 \pm 0.23$  pmoles respectively,  $P < 0.05$ , t-test).

## **Discussion**

In the present study, we showed that an enzyme capable of degrading oxytocin (P-LAP) is expressed in the magnocellular oxytocin neurons of the SON and PVN, the main location of oxytocin synthesis. We found that P-LAP mRNA expression in the PVN is significantly increased during lactation, a time when extracellular oxytocin plays a key role in regulating milk ejection and maternal behavior, and this increase is accompanied by increases in the hypothalamic and posterior pituitary gland activity of leucyl and cystinyl aminopeptidases.

It seems unlikely that the measured aminopeptidase activity in posterior pituitary and hypothalamic extracts is solely attributable to P-LAP, because of the apparent tissue specific divergence in the leucyl aminopeptidase activity and cystinyl aminopeptidase activity. Nevertheless, P-LAP evidently plays an important role in regulating central oxytocin availability, including regulating of the pattern of oxytocin neuronal firing during lactation. However, we did not find a significant increase in either P-LAP mRNA in the SON or PVN, nor in hypothalamic P-LAP activity in late-pregnant rats. This is perhaps not unexpected given that the oxytocin neurons are relatively quiescent in late pregnancy (which is proposed to minimise the risk of preterm birth) via an endogenous inhibitory opioid mechanism induced by allopregnanolone (29, 34, 35). The present data also indicate that altered hypothalamic P-LAP activity/expression in pregnancy is not involved in preventing preterm birth. However, it is likely that the action of P-LAP at other sites e.g. the uterus does play an important role given that the onset of labour is advanced in P-LAP knockout mice (12).

Oxytocinase enzyme activity in the hypothalamus was consistently higher in lactating rats than in virgins, and it seems likely that this alters the regulation of central oxytocin actions. P-LAP mRNA expression was also higher in the PVN in lactation, but we did not observe a similar increase in the SON. However, most of the neurons in the SON make vasopressin, not oxytocin, whereas oxytocin is relatively more abundant in the PVN (36), and as P-LAP is expressed in both oxytocin neurons and vasopressin neurons, it may simply be that an increase in P-LAP expression in SON oxytocin neurons is hidden within a larger signal from vasopressin neurons (37).

The immunocytochemical studies were consistent with changes in P-LAP trafficking in oxytocin neurons, but do not provide evidence of expression level. P-LAP immunoreactivity was present in the somata and dendrites of all oxytocin neurons, and *in vitro* enzyme activity was measurable in the posterior pituitary, where the axons terminate. Using double immunofluorescence we observed punctate labelling of P-LAP within neurons, which suggests that it is packaged in vesicles, consistent with reports of P-LAP packaged in GLUT4- containing vesicles in peripheral tissues (38, 39). Colocalization of the enzyme with the Golgi marker GM130 was high, and particularly strong in the perinuclear region of the cell where the Golgi apparatus is located. This is also consistent with reports of a population of GLUT4 vesicles containing P-LAP being tethered at the Golgi apparatus by the protein tankyrase (40).

Although degradation of oxytocin in the brain has been studied previously (41), the sites, rate and regulation of degradation have been poorly characterized. As oxytocin concentrations are generally higher in the CSF than in plasma, and are present there at physiologically active concentrations, it is clear that a significant amount of centrally released oxytocin evades local degradation to diffuse (or be conveyed) from its release sites in the brain to the ventricles. Oxytocin in the CSF has a half-life of about 20 min, indicating that its

clearance from CSF is mainly by bulk flow rather than enzymatic degradation (42). However, in the CSF, concentrations of oxytocin-associated neurophysin exceed those of oxytocin by a factor of about 50, although these are co-secreted in equimolar amounts. As neurophysin is considered to not be degraded (41), there is clearly substantial degradation of oxytocin en route through the extracellular space to the CSF.

Finally, blocking central oxytocinase action revealed a physiological role for oxytocinase in controlling the milk-ejection reflex, indicating that degradation of oxytocin contributes to the control of oxytocin availability to its receptors within the hypothalamus. These data extend previous reports that blocking aminopeptidase action in vitro modifies oxytocin neuron electrical activity (23). Oxytocin has important effects on a variety of social behaviours (43), and it has been widely suggested that it may be a valuable therapeutic target in treating autism (44). Furthermore, decreased oxytocinase activity in the amygdala following stress indicates a role in modulating oxytocin actions in the context of stress responses (45). Thus our present observations highlight the possibility of targetting central oxytocin actions by targetting the enzymes that are active in its degradation.

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## Figure Legends

### **Figure 1.**

**A,B)** Double immunofluorescent labelling for P-LAP (green) and oxytocin-neurophysin (red) in the PVN of a virgin female rat. The merged images (**C,D**) reveal co-localization (orange, arrow lines) of P-LAP in oxytocin-neurophysin neurons and dendrites and presence in adjacent neurons (hollow arrows). Scale bars A-C: 100 $\mu$ m, D: 20 $\mu$ m.

### **Figure 2.**

Images of cellular localization of either (**A**) oxytocin-neurophysin (red) or (**B**) GM130 (Golgi marker, red) with P-LAP (green) and phalloidin (F-actin marker, blue) and merged images in SON neurons from virgin, pregnant and lactating rats. Note that P-LAP is also found in presumptive vasopressin neurons (VP). Quantification of covariance data of P-LAP and oxytocin-neurophysin (**C**) or GM130 (**D**) using Pearson's coefficient values from SON neurons in virgin, pregnant and lactating rats (mean  $\pm$  SEM, N=15 per group) Intracellular distribution of oxytocin-neurophysin (**E**) or P-LAP (**F**) in the treatment groups. Scale bars = 10 $\mu$ m, \* $P$ <0.05 compared to virgin control, one-way ANOVA followed by Tukey's post-hoc test.

### **Figure 3.**

**A)** Example images of an oxytocin neuron (from a lactating rat) with immunoreactivity for oxytocin-neurophysin (red) and P-LAP (green). The outline of the cell (drawn in blue) was defined using the phalloidin signal. The yellow line is an example of one of the 16 lines drawn from the nucleus to plasma membrane and used to measure the signal intensity of each

channel along each line (shown here in white above the line) (N=15 cells/group). Scale bar = 5 $\mu$ m. **B)** The signal intensities for each channel within 1 $\mu$ m either to the nucleus or the plasma membrane are expressed as a percentage of the total of each line and the averages plotted for oxytocin and P-LAP from the three groups. \* $P$ <0.05 compared to virgins, one-way ANOVA followed by Tukey's post-hoc test.

#### **Figure 4.**

**A)** Light photomicrographs of film autoradiographs of placenta processed for P-LAP mRNA by *in situ* hybridization showing the antisense (left) and sense control (right). **B)** Light photomicrographs of autoradiographs of the hypothalamus showing hybridization of P-LAP mRNA in the SON and PVN. Quantification of grain density of P-LAP mRNA in the **(C)** SON and **(D)** magnocellular sub-division of the PVN from virgin, pregnant and lactating rats (mean  $\pm$  SEM). Scale bars = 200 $\mu$ m. One way ANOVA followed by Tukey's post-hoc test, \* $P$ <0.05 vs. virgin and pregnant groups.  $F=5.841$ , d.f. = 16.  $N= 6, 6$  and  $5$  for virgin, pregnant and lactating groups respectively.

#### **Figure 5.**

Aminopeptidase activity in soluble and membrane-bound fractions of **(A)** hypothalamus or **(B)** posterior pituitary tissue from virgin, pregnant and lactating rats (mean  $\pm$  SEM,  $n=6-7$  per group). One-way ANOVA followed by Tukey's post-hoc test, \* $P$ <0.05 vs virgins.

#### **Figure 6.**

Examples of intramammary pressure (i.m.p.) recordings of the milk-ejection reflex (MER) before and after i.c.v. **(A)** oxytocin (1pmole in 2 $\mu$ l) or **(B)** amastatin (P-LAP inhibitor; 6 nmole in 2 $\mu$ l). Sharp increases in i.m.p. during suckling reflect milk ejections caused by

action of a pulse of oxytocin secreted by the posterior pituitary as a result of synchronised burst-firing of magnocellular oxytocin neurones. Burst-firing, and hence milk ejections are facilitated by auto-excitatory actions of oxytocin released in the SON and PVN: thus i.c.v. oxytocin triggers milk ejections, and amastatin acts similarly. Scale bar 10min. C) The frequency, but not the average amplitude, of the MER after i.c.v. oxytocin or amastatin was significantly greater ( $P < 0.05$ , t-test). Using a dose-response curve of the amplitude of intramammary pressure changes in response to known concentrations of oxytocin given i.v., there was a significant increase in oxytocin release after i.c.v. injection of oxytocin or amastatin ( $P < 0.05$ , t-test,  $n=5$ ).

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