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Abstract

In the main olfactory system, odours are registered at the main olfactory epithelium, then processed at the main olfactory bulb (MOB) and subsequently by the anterior olfactory nucleus (AON), the piriform cortex (PC) and the cortical amygdala. Previously, we reported populations of vasopressin neurons in different areas of the rat olfactory system, including the MOB, accessory olfactory bulb (AOB) and the AON and showed that these are involved in the coding of social odour information. Utilizing immunohistochemistry and a transgenic rat in which an enhanced green fluorescent protein reporter gene is

expressed in vasopressin neurons (eGFP-vasopressin), we show here a population of vasopressin neurons in the PC. The vasopressin neurons are predominantly located in the layer II of the PC and the majority co-express the excitatory transmitter glutamate. Furthermore, there is no sex difference in the number of neurons expressing vasopressin. Electrical stimulation of the lateral olfactory tract (LOT) leads to a significant increase in the number of Fos-positive nuclei in the PC, MOB, AOB, dorsal AON, and supraoptic nucleus (SON). However, there was only a significant increase in Fos expression in vasopressin cells of the PC and SON. Thus functionally distinct populations of vasopressin cells are implicated in olfactory processing at multiple stages of the olfactory pathway.

Abbreviations: AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; BNST, bed nucleus of the stria terminalis; MOB, main olfactory bulb; LOT, lateral olfactory tract; PC, piriform cortex; SON, supraoptic nucleus

Keywords: anterior olfactory nucleus, main olfactory bulb, accessory olfactory bulb, supraoptic nucleus

Running title: Vasopressin and the piriform cortex

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INTRODUCTION

Olfactory signals, including those of social odour cues, have powerful behavioural effects in many species, including man. The processing of olfactory cues in mammals is handled by two anatomically distinct pathways: the main, and the accessory olfactory systems (1-3). In the accessory olfactory system, pheromones are received at the vomeronasal organ, then processed at the accessory olfactory bulb (AOB) and then higher brain regions, most importantly the medial amygdala (4). In the main olfactory system, odours are registered at the main olfactory epithelium, then processed at the main olfactory bulb (MOB) and subsequently by the anterior olfactory nucleus (AON), the piriform cortex (PC) and the cortical amygdala (5, 6). The PC is more than a primary olfactory relay and plays an active role from sensory to more cognitive aspects of odour perception.

71 The neuropeptide vasopressin, produced in the hypothalamus and secreted from
72 the pituitary, has a key role in electrolyte and fluid homeostasis, but recent interest in
73 vasopressin has been dominated by its functions within the brain and, especially on its
74 involvement in social recognition. Vasopressin is involved in the modulation of social
75 recognition at the level of the olfactory bulbs (7, 8) and brain regions such as the lateral
76 septum (9, 10). The neuropeptide also regulates social behaviours such as aggression (11,
77 12), pair-bonding (13, 14), and parental behaviour (15, 16) and has also been linked to
78 human social behaviours in health (17-19) and during neurological disorders such as
79 autism (20, 21).
80 Previously, we reported that the rat olfactory system contains several population
81 of interneurons which express vasopressin. They are localized in the MOB, AOB and
82 anterior AON (7, 22). Vasopressin V1a receptors are expressed in the MOB and AON (7,
83 22-26) and V1b receptors in the AON, olfactory tubercle and PC (22, 27) which suggests
84 that these cells might be sensitive to their own signal. Blocking the actions of vasopressin
85 in the MOB impairs the social recognition abilities of rats and vasopressin agonists and
86 antagonists can modulate the processing of information by olfactory bulb neurons (7, 8).
87 Furthermore, exposure of adult rats to a conspecific juvenile, but not to a heterospecific
88 predator odour, increases early growth response protein 1 (Egr-1) expression in
89 vasopressin neurons of the AON (22). These data suggest that vasopressin neurons in the
90 olfactory system are involved in the coding of social odour information (28, 29).
91 Here we describe a population of vasopressin neurons distributed across the
92 length of the PC. As previously, by utilizing a transgenic rat line in which an enhanced
93 green fluorescent protein reporter gene is expressed specifically in vasopressin cells
94 (eGFP-vasopressin) (30) we characterised these neurons based on a number of other

chemical markers and determined whether there are sex differences in the number of vasopressin-expressing cells in the PC. Finally, to understand how the inputs to the olfactory system (OS) influence vasopressin neurons in the different parts of the olfactory system and the hypothalamus, we electrically stimulated the lateral olfactory tract (LOT) and measured the expression of the immediate early gene c-*fos* by immunocytochemical detection of Fos, the protein product of c-*fos*, in wild type and transgenic rats.

MATERIALS AND METHODS

Ethical Approval

Procedures conducted in the UK were approved by the local Ethics Committee and the UK Home Office under the Animals Scientific Procedures Act 1986. Experiments were performed on adult male and female wild-type Sprague-Dawley and transgenic rats (250-450 g), housed under controlled conditions (12 h light: 12 h dark, 21^{o}C) with free access to food and water. Most of the immunohistochemistry was carried out on a homozygous line of transgenic rats expressing a vasopressin-eGFP (enhanced green fluorescent protein) fusion gene (30).

LOT stimulation

Rats were anaesthetised with an i.p. injection of sodium pentobarbital (60 mg/kg) and the level of anaesthesia was monitored throughout the surgical procedure. Supplementary doses were administered as necessary.

The rats were prepared for dorsal surgery, and holes were drilled over both the left and right LOT. A concentric stainless steel electrode (100 µm tip diameter, SNEX-100, Clark Electromedical Instrument, Kent, UK) was lowered into the LOT (below the

piriform cortex, 1.4 mm posterior to bregma and 3.2 mm lateral to midline, 9.5 mm deep) of the right hemisphere to deliver a biphasic pulse (1 mA peak-to-peak with a width of 1 ms) at 50 Hz for 10 min generated by a GRASS S88 stimulator with stimulus isolation and constant current units (Grass Products, Warwick, USA). This stimulus intensity was used to overwrite the spontaneous output of the MOB neurons; mitral cells show a firing rate up to 30 spikes/s (7, 31). Furthermore, recordings of the spontaneous activity of mitral cells have shown that mitral cells fire in a phasic discharge pattern, with periods of activity lasting on average 2 min with similar periods of quiescence between these bursts (31). Taking this into account, a constant stimulation over a prolonged period of time was applied, to disrupt the spontaneous output pattern.

The electrode was then removed and placed in the LOT of the left hemisphere for 10 min without any electrical stimulation. For the control group, the electrode was placed to the both sides of the LOT for 10 min without stimulation. To mimic the activation throughout the olfactory system by the input, we applied 10 min of 50 Hz electrical stimulation to the LOT and analysed the expression of Fos. At 90 min after the end of electrical stimulation, rats were terminally anesthetized and transcardially perfused for tissue collection. The position of the stimulating electrodes in LOTs was verified histologically (Fig. 4F).

Tissue preparation

Rats were terminally anesthetized (isoflurane inhalation then sodium pentobarbital, 200 mg/kg body weight, i.p.) and transcardially perfused with a heparinised (20 U/ml) 0.9% saline solution followed by paraformaldehyde (PFA) 4% in 0.1 m phosphate buffer (PB). The brains were removed and immersed overnight in a solution

of 2% paraformaldehyde and 15% sucrose in 0.1 M PB at 4ºC. Then the brains were placed in a solution of 30% sucrose in 0.1M PB and left for at least 72 h before they were processed. The rat brains were cut using a freezing microtome and stored in a cryoprotectant solution (30% ethylene glycol + 20% glycerol in 0.05 M sodium phosphate buffer, pH 7.3) at 4°C until required.

Immunocytochemistry

Immunochemistry on free-floating sections was performed for both wild type and transgenic rats. For wild-type rats, coronal sections were cut at 40 μm and washed in PB + 0.2% Triton X-100 for 3 times to remove excess fixative/cryoprotectant. Sections were then given a 5-min wash in PB before blocking endogenous peroxidase using the methanol solution (PB + 20% v/v methanol + 0.3% w/v hydrogen peroxide). Again, the sections were washed with PB+ 0.2% Triton X-100 three times for 5 min. To block non-specific staining, sections were incubated for 30 min in a PB blocking buffer consisting of 1% normal sheep serum + 0.3% Triton X-100. The sections were incubated in the c-Fos antibody (Ab-2, Oncogene Sciences, Cambridge Bioscience, UK) at 1:1000 dilution in PB blocking buffer for an optimum of 36 h at 4°C. After sections were washed in PB + 0.2% Triton X-100 for three times, they were incubated for 60 min with Biotinylated-anti-rabbit IgG (1:100, Vector Laboratories, Inc., Peterborough, UK) in PB+ 3% normal goat serum+0.2% Triton X-100 at room temperature. Sections were next incubated for 60 min in ABC complex diluted as detailed by the manufacturer (Vectorstain elite ABC kit, Vector Laboratories, Inc., Peterborough, UK). The sections were rinsed twice in PB + 0.2% Triton X-100, followed by 0.2M acetate buffer (pH 6.0) and visualized by incubating the sections in the glucose oxidase-Ni DAB (3, 3'- diaminobenzidine) solution

(0.025% DAB + 2.5% Nickel II sulphate + 0.08% ammonium chloride + 0.4% β-D-Glucose + 0.003% Glucose oxidase + 0.2M acetate buffer).

Brain tissues from transgenic rats were cut as 40-μm sagittal sections and double immunochemistry was performed for Fos and eGFP as previously described (7, 32). Briefly, after washing in 0.1M PB to remove all cryoprotectant, sections were incubated for 20 min in 0.3% H_2O_2 in 0.1M PB to quench endogenous peroxidase activity. Sections were washed at least four times with 0.1 M PB + 0.3% Triton X-100 between each of the following steps unless otherwise specified. To block non-specific interaction of secondary antibodies with the tissue, sections were then incubated for 60 min in a blocking buffer consisting of 3% normal goat serum + 0.3% Triton X-100 in 0.1M PB. Then sections were incubated for 48 h at 4 °C in c-Fos polyclonal antibody raised in rabbit diluted in the blocking buffer (1:30,000, Synaptic systems, Goettingen, Germany). After extensive washing in 0.1M PB, the sections were incubated for 60 min with biotinylated-anti-rabbit IgG (1:100, Vector Laboratories, Inc., Peterborough, UK). Sections were next incubated for 60 min in ABC complex diluted as detailed by the manufacturer (Vectorstain elite ABC kit, Vector Laboratories, Inc., Peterborough, UK) Fos immunoreactivity was visualized using a solution of 0.025% DAB + 2.5% Nickel II sulphate + 0.08% ammonium chloride + 0.015% H_2O_2 in 0.1 M Tris. For eGFP immunostaining, sections were incubated in chicken anti-GFP polyclonal antibody (Abcam, Cambridge, UK) diluted 1:5000 for 48 h at 4 °C. The sections were then incubated for 1 h in Biotinylated-anti-chicken IgG (1:100, Vector Laboratories, Inc., Peterborough, UK). To visualise the eGFP immunoreaction, a solution containing 0.025% DAB and 0.015% H_2O_2 in 0.1 m Tris was used. All the sections from wild-type and transgenic rats were mounted on the gelatinised slides and air dried. After dehydration in

ascending concentrations of ethanol (70% and 90% for 5 min each then 95% and 2 x 100% for 10 min each and then Xylene 2 x 10 min), slides were cover slipped using DPX mountant.

For immunohistochemistry involving exposure to a biotinylated secondary and fluorescently tagged streptavidin, sections were blocked for endogenous biotin by incubating them first in 0.01% avidin in 0.1 M PB for 30 min, washing and then incubating in 0.001% biotin in 0.1 M PB for 30 min. After washing, sections were incubated for 60 min in a blocking buffer consisting of 3–5% normal serum (matched to the host of secondary animal) + 0.1% Triton X-100 diluted in 0.1 M PB. The sections were incubated with primary antibodies (Table 1) diluted in the blocking buffer. The primary antibodies were applied for 1–5 days at room temperature for the first day and thereafter at 4°C. After washing in 0.1 M PB, sections were incubated for 60 min with the appropriate secondary antibody (Table 2) and then washed in 0.1 M PB. Sections exposed to biotinylated secondary antibodies were then incubated for 60 min with fluorescently labelled streptavidin conjugate (1:500). Both secondary antibodies and fluorescently labelled streptavidin were diluted in 0.1 M PB + 0.03% Tween20. After further washing, sections were incubated in 4,6-diamidino-2-phenylindole (DAPI, 1:33 000, Life Technologies Ltd, UK) for 5 min at room temperature, washed and coverslipped using Permafluor Aqueous Mounting Medium (Thermofisher scientific, Waltham, MA, USA). No fluorescent labelling was detected when primary antibodies were omitted or when the primary antibodies (Table 2) were incubated with a fivefold (w/v) control immunogen before being exposed to the tissue sections (the latter control was conducted whenever a control peptide was available from the supplier of that primary antibody).

Microscopy

Fluorescence signals were acquired either using a Nikon AIR confocal or a Zeiss LSM510 Axiovert confocal laser scanning microscope. In either case, the images were acquired at 1024x1024 pixels, using a Nikon Plan Apochromat 1.4 NA x 60 oil immersion objective or a Zeiss Plan NeoFLUAR 1.4 NA x 63 oil-immersion objective respectively. In all cases, emissions for each fluorophore were obtained consecutively to avoid channel cross-talk. Those images taken throughout each cell at Nyquist sampling rates were deconvolved using Huygens software (Scientific Volume Imaging, Hilversum, Netherlands) and all images were analysed using NIH ImageJ software (v1.48) and figures constructed using Microsoft PowerPoint.

Cell count

Immunohistochemically stained sections were imaged (Leica DFC490, 20x objective) for subsequent cell counting. Images were taken consecutively across the length of the PC. Seven sagittal sections from each of 12 brains (n=6/group) of eGFP-AVP transgenic rats, immunostained for GFP, were used to determine whether there are sex differences in the number of vasopressin expressing cells in the PC. We also analysed sections of the bed nucleus of the stria terminalis (BNST; three sections from each rat), since it has previously been shown that there is a sexual dimorphism in the number of vasopressin expressing cells in this area (33, 34).

Quantification of Fos positive cells

The number of Fos positive cells was quantified in a number of brain regions, including the PC, MOB, AOB, AON and SON. The investigators were blinded to the

239 treatments at the time of counting. The images were acquired using a Leica upright
240 microscope, ×10 objective and digital camera and the Leica system acquisition software
241 (Leica Microsystems, Wetzlar, Germany). For counting Fos and GFP positive nuclei, we
242 carried out the counting directly under the microscope using × 20 magnification to avoid
243 miscounting areas in which neurons are densely packed. For each brain region, counts
244 were made on at least 3 sections for each animal and the mean values (± SEM) were
245 calculated. For counting Fos positive nuclei in the mitral cell layer in wild-type rats,
246 coronal sections are used (6.7 mm anterior to bregma). For counting Fos expression in
247 vasopressin cells we used vasopressin-eGFP transgenic rats. Counting was conducted in
248 saggital sections (1.4-1.9 mm lateral to the midline) including the MOB, AOB, AON,
249 SON. For counting in the piriform cortex, saggital sections 3.4 - 3.9 mm lateral to the
250 midline were used (35).

251 Using ImageJ (NIH, Bethesda, MD, USA), acquired images were converted to 8-
252 bit, thresholded using the same parameters, and Fos-positive nuclei were counted
253 manually using the Cell Counter tool. The number of Fos-positive nuclei within each
254 region of interest (ROI) was normalised to the area of that ROI to allow comparison
255 (mean ± SEM/10^4 μm^2), except for the PC. The total number of Fos-positive nuclei was
256 counted and is expressed as mean ± SEM per section. Vasopressin neurons are densely
257 packed in some regions and cell structures in the sections overlap, sometimes making it
258 hard to distinguish between single neurons, which might affect counting Fos-positive
259 nuclei in vasopressin stained neurons. Therefore, we also counted a proportion of clearly
260 distinguishable vasopressin neurons and vasopressin neurons expressing Fos protein
261 directly under the microscope (x 20 magnification in at least six regions in every rat, and
262 the values are expressed as percentages).

Statistics

Statistical analysis was performed using the Prism software. To compare data between two independent groups the Mann-Whitney Rank Sum Test was used. All data are shown as mean ± SEM.

RESULTS

Characterization of eGFP-vasopressin neurons in the PC

We first discovered these cells in our transgenic rat strain in which eGFP is expressed under the control of the vasopressin promoter (Fig. 1A,B). In these transgenic rats, eGFP expressing cells were distributed widely throughout the whole PC. Using double immunocytochemistry, we established that every cell immunoreactive for eGFP was also immunoreactive for vasopressin (Fig. 1B). We confirmed the expression of vasopressin in PC cells in wild-type rats using antibodies against vasopressin-neurophysin (Fig. 1C). Whereas vasopressin cells were seen across the whole PC, the signal was less intense and the number of vasopressin immuno-reactive cells in wild-type rats was lower than seen in eGFP-vasopressin transgenics.

Most of the vasopressin-immunoreactive cells were localised in layer II of the PC, however a few were also seen in layer Ib and layer III (Fig. 1B); 63% of these cells co-expressed glutamate (140 of 221 examined eGFP cells, Fig. 1D) and 20% co-expressed GABA (56 of 257, Fig. 2). In very few cells, eGFP was co-localised with calbindin (10.5%, 21 of 200) and calretinin (0.7%, 2 of 287), but not parvalbumin (0 of 323, Fig. 2), which label known subpopulations of PC neurons (36-38). There was no co-

localisation with cholecystokinin (CCK, 0 of 423) or vasoactive intestinal polypeptide (VIP, 0 of 323) which have been described in neurons of the PC (Fig. 2) (36, 39).

Vasopressin receptor staining was abundant in the PC, but very few eGFP-vasopressin cells were immunoreactive for V1a (6%, 22 of 365) and V1b receptors (1.8%, 3 of 163, Fig. 2).

Sex differences

To determine whether there are sex differences in the number of vasopressin expressing cells in the PC as described for other areas harbouring vasopressin cells (33, 40), sagittal sections from eGFP-AVP transgenic rats were immunostained for GFP. There was no significant difference between male and female rats in the number of immunoreactive vasopressin cells in the PC (males 533 ± 71, females 589 ± 127; P=0.71, Students t-test, n =6/group, Fig. 3B,C). By contrast, as expected, in the BNST there were fewer immunoreactive vasopressin cells in female rats than in male rats (females 105 ± 15, males 204 ± 31, n = 4/group, P = 0.04; Fig. 3D).

Fos induction after LOT stimulation

Initially we studied the effects of electrical stimulation of the LOT on Fos induction in the MOB mitral cell layer in coronal sections from wild-type SD rats. The number of Fos-positive neurons was higher in the mitral cell layer ipsilateral to LOT stimulation compared to the controls (61.6 ± 13.1 vs 20.7 ± 3.7 nuclei/section, p=0.02). There was no significant difference in Fos expression in the mitral cell layer on the contralateral side (contralateral stimulated, 11.0 ± 5.8 cells/section vs control, 17.2 ± 2.5 cells/section). To determine the effects of LOT stimulation on Fos expression specifically

310 in vasopressin neurons, subsequent studies were performed in vasopressin-eGFP
311 transgenic rats.
312
313 *Main and accessory olfactory bulb*
314 We repeated this experiment and analysed sagittal sections of the MOB, AOB,
315 AON and PC. LOT stimulation again increased Fos expression in the ipsi-, but not
316 contralateral side in the mitral cell layer of the MOB (ipsilateral, 12.9 ± 2.2, vs 2.4 ± 0.8
317 cells/10^4 μm^2, p = 0.001; contralateral, 2.8 ± 1.3 cells/10^4 μm^2 vs 2.0 ± 0.2 cells/10^4 μm^2,
318 Fig. 4A). In the AOB, the number of Fos-positive nuclei in the mitral cell layer following
319 LOT stimulation was also significantly higher in the ipsilateral side than on the ipsilateral
320 side of the control rats (6.7 ± 0.8 vs 3.7 ± 1.0 cells/10^4 μm^2, p = 0.041, Fig. 4B). An
321 equivalent amount of Fos was induced in the contralateral mitral cell layer in response to
322 LOT stimulation, however this failed to reach statistical significance compared to the
323 control group (stimulated contralateral 6.8 ± 0.9 cells/ 10^4 μm^2 vs control contralateral,
324 4.1 ± 0.9 cells/ 10^4 μm^2, p = 0.1091, Fig. 4B).
325 There are no vasopressin expressing cells in the mitral cell layer of the MOB and
326 very few in the AOB. The proportion of vasopressin neurons expressing Fos protein in
327 the mitral cell layer of the ipsilateral AOB in response to LOT stimulation was 2.5 ± 0.5%
328 (no Fos protein expression was observed in the contralateral side and in the control
329 unstimulated animals).
330
331 *Anterior olfactory nucleus*
332 In the AON, we separately analysed the dorsal and lateral parts of the AON (22).
333 The number of Fos-positive nuclei differed between the ventral and dorsal part of the

AON, but this did not reach statistical significance (control-ipsilateral of ventral vs dorsal, 11.8 ± 4.7 cells/10^4 μm^2 vs 11.8 ± 4.7 cells/10^4 μm^2, p = 0.051, 4.7-5.2 mm anterior to bregma).

In the *dorsal* AON, LOT stimulation induced a significant increase in the number of Fos-positive nuclei in the ipsilateral side compared to the control group (5.7 ± 1.4 cells/10^4 μm^2 vs control 1.1 ± 0.4 cells/10^4 μm^2, p = 0.019, Fig. 4C). In the *ventral* AON, there was no significant difference in the levels of Fos expression between the control and LOT stimulated group on either side (stimulated ipsilateral, 8.4 ± 3.6 cells/10^4 μm^2 vs control ipsilateral, 11.8 ± 4.7 cells/10^4 μm^2, p = 0.53).

There is a large number of vasopressin-expressing cells in the AON, but there was no significant change in the proportion of vasopressin cells expressing Fos in response to LOT stimulation in both groups ipsi- and contralateral (*dorsal*, control vs stimulated, 3.8 ± 1.9 vs 5.1 ± 1.9 %, p=0.90 , ventral, 14.5 ± 3.9 vs 7.0 ± 2.4 %, p=0.44) and contralateral (dorsal, control vs stimulated, 2.0 ± 1.4 vs 1.2 ± 0.4 % , p=0.90 , ventral, 10.0 ± 1.8 vs 9.8 ± 1.7 %, p=0.72, Fig.4G).

Piriform cortex

The PC runs in a band from the rhinal fissure to the ventral surface of the brain following the curvature of the lateral wall. LOT stimulation induced a striking increase in Fos expression in the PC (Fig. 4D). Following LOT stimulation 253 ± 61 cells/section expressed Fos in the ipsilateral side, compared to 59 ± 12 cells/section in control rats (p = 0.042). LOT stimulation also significantly increased the number of Fos-positive nuclei in the contralateral side indicating a bilateral connection between the two hemispheres of

the olfactory system (stimulated contralateral, 252.2 ± 80.9 cells/section vs control ipsilateral 58.8 ± 12.3 cells/section, p = 0.029, Fig. 4D).

There was also a significant increase in the proportion of Fos-positive eGFP cells in the PC in the electrically stimulated ipsilateral side compare to control (stimulated ipsilateral 37 ± 7%, vs control ipsilateral 6.0 ± 0.8%, p = 0.02, Fig. 4H).

Supraoptic Nucleus

Connectivity between the SON and the olfactory system has been described previously (41-44). Therefore, we also analysed Fos induction after electrical stimulation of the LOT in the SON (Fig. 5A). There was a significant difference between the control and the electrically stimulated group in both hemispheres (stimulated ipsilateral 10.0 ± 1.3/10^4 μm^2 vs control ipsilateral, 2.3 ± 0.7/10^4 μm^2, p = 0.02, stimulated contralateral, 6.9 ± 0.8 /10^4 μm^2 vs control ipsilateral, 2.3 ± 0.7/10^4 μm^2, p = 0.03, Fig. 5B).

There was a significant increase in the proportion of Fos-positive eGFP cells in the LOT stimulated ipsilateral and contralateral side compare to control (stimulated ipsilateral 63.7 ± 5.8%, stimulated contralateral 54.5 ± 4.8% vs control ipsilateral 18.7 ± 6.7%, p = 0.02, 0.03, Fig. 5C).

DISCUSSION

Here we show a large number of cells expressing vasopressin in the PC. The PC has been characterised both electrophysiologically and immunocytochemically (45-47) and comprises a sparsely populated superficial layer I, a main input layer II containing the densely-packed somata of glutamatergic principal neurons, and a deep layer III containing principal neurons at lower density (48). Most of the vasopressin cells in the

PC are located in layer II and also co-express glutamate. Scattered more uniformly across all layers are GABA-releasing interneurons that provide feedforward or feedback synaptic inhibition to principal cells and some of these also contain vasopressin. Of the vasopressin cells in the PC, 30% showed an increase in Fos expression after LOT stimulation. These cells are likely to be glutamatergic pyramidal neurons since most cells which receive direct bulbar inputs in layer II are pyramidal neurons (48).

We first discovered these cells in our transgenic rat strain in which eGFP is expressed under the control of the vasopressin promoter. These rats have been extensively used by us and others to study vasopressin-expressing neurons in distinct brain regions (7, 22, 32, 49, 50). The vasopressin-eGFP transgene encodes a modified vasopressin precursor with eGFP fused in-frame at the C terminus ((30) and D. Murphy, personal communication). The signal peptide, vasopressin and neurophysin portions of the precursor are intact, and may be expressed from the transgene, thus the vasopressin-associated neurophysin in eGFP rats may reflect either endogenous expression or transgene-driven expression. We therefore confirmed the expression of vasopressin in PC cells in wild-type rats using antibodies against vasopressin-neurophysin.

Here we confirmed the presence of vasopressin receptor expression in the PC (22, 27), however the receptors are not on the vasopressin cells themselves, indicating that the neuropeptide is not involved in autoregulation as it is in the hypothalamus (51, 52). The PC is part of a network involved in the processing of olfactory cues used for social communication (53-55) integrating odour features into odour objects (56). We and others have previously shown that vasopressin is involved in the modulation of social recognition at the level of the OB (7, 8) and the AON (22), suggesting that vasopressin neurons in the olfactory system are involved in the coding of social odour information

405 (28, 29). It is believed that vasopressin and the closely related neuropeptide oxytocin
406 modify the state of early olfactory presentation to enhance salience of concurrently-
407 presented odours and to help detect relevant information of conspecifics during social
408 encounters. This is supported by experiments showing longer conspecific exploration
409 times in rodents where the oxytocin receptor was deleted in the AON, or vasopressin
410 receptor blocked or downregulated in the MOB (7, 57). These animals may have shown
411 less efficient information extraction due to peptide effects on the gain of odour
412 representations. Through cortical top-down projections into the olfactory system, the
413 peptides may modify the global gain control of olfactory coding before MOB outputs
414 spreads into divergent higher-order pathways including the PC, the ventral striatum
415 (olfactory tubercle), the amygdala, and the entorhinal cortices (58, 59). Many of these
416 higher-order brain regions are activated during social interactions and also express
417 oxytocin and vasopressin receptors, allowing for further modifications of information
418 through these peptides during particular types of social behaviour. It is also likely that
419 storage of recognition memory involves brain regions like the PC. Oxytocin for example
420 promotes formation of association learning of an initially neutral odour with a potential
421 mating partner (60). Activation of the PC was found to be crucial for the consolidation
422 and for the recall of long-term social memory (61). However, the exact role of vasopressin
423 in these processes has still to be determined in more detail.
424
425 **Lack of sex differences**
426 Here we show that there was no sex difference in the number of vasopressin
427 expressing cells in the PC, but that there was in the BNST. A lack of sexual dimorphism
428 has also been shown in other vasopressin cell populations of the olfactory system,

429 including the MOB, AOB or the AON (7, 22). By contrast, immunoreactive vasopressin
430 projections in the lateral septum and the lateral habenular nucleus are much denser in
431 male than in female rats (40) and a similar imbalance in peptide expression was identified
432 in the BNST (33, 62). The role of vasopressin in modulating social behaviours is different
433 in male and female rodents. Vasopressin V1a receptor knock-out female mice display
434 significantly less anxiety-related behaviour than male equivalents (9) and variations in
435 maternal care is influenced by the expression of oxytocin and vasopressin receptors in the
436 lateral septum and amygdala in a gender-specific manner (63); both areas show sex
437 dimorphic vasopressin expression (40).
438
439 **Fos expression in response to LOT stimulation**
440 LOT stimulation increased Fos expression in the ipsilateral, but not in the
441 contralateral side, of the mitral cell layer of the MOB and the AON. There are no direct
442 inter-bulbar connections, and no direct connections to the contralateral cortex regions
443 from mitral/tufted cells of the MOB (64). However, the increase in Fos expression in the
444 AOB cannot be explained by the direct activation through LOT stimulation. The
445 vomeronasal system has separate circuitries and the mitral/tufted cells of the AOB project
446 through the accessory lateral olfactory tract to the bed nucleus of stria terminalis (BNST),
447 nucleus of accessory olfactory tract, and the medial portion of the amygdala (vomeronasal
448 amygdala) from which tertiary projections target certain regions of the hypothalamus (65,
449 66). Whether the increase in Fos expression in the AOB in response to LOT stimulation
450 reflects activation of feed forward connections to the AOB needs to be determined in
451 more detail.

We observed a significant increase in Fos expression in the contralateral side of the PC following stimulation. The cortical feedback projection from PC to olfactory bulb is complex and direct bilateral connections between PC have not yet been shown. However, an ipsilateral projection from the anterior PC to the pars lateralis of AON has been demonstrated (67) and thus the information transfer may occur to the contralateral PC through the AON (68).

Fos labelling in the SON

In our experiments, LOT stimulation increased Fos expression in the SON. Connectivity between the SON and the olfactory system has been described previously (41-44, 69). Labelling studies have shown a monosynaptic pathway between the olfactory bulb and the SON (42, 43, 70) and connections between olfactory sensory neurons and the vasopressin neurons in the PVN and SON (44). In addition, Hatton and Yang performed electrophysiological recordings *in vitro* and upon electrical stimulation of the LOT they found that cells within the SON responded with short latency excitatory responses supportive of a direct pathway from the olfactory bulb to the SON (41). Recent studies indicate a role for oxytocin in the connections between the olfactory bulb and PVN in the context of social interaction (57, 60). However, besides studies showing anatomical connectivity between olfactory bulb and SON and PVN, the interplay between these connections and the role of vasopressin needs to be determined in more detail.

Taken together, populations of vasopressin neurons in different areas of the rat olfactory system, including the MOB, AOB and the AON have been shown to be involved in the coding of social odour information (7, 22). Here we describe an additional population of

vasopressin cells in the PC. Together these functionally distinct populations of vasopressin cells in different parts of the olfactory system suggest that vasopressin signalling is involved in information processing at multiple levels of the olfactory pathway.

FIGURE LEGENDS

Figure 1: Vasopressin neurons in the piriform cortex.

A, Coronal section from rat atlas indicating area of piriform cortex (PC) where images in (**Aii, Aiii**) have been taken from, showing that eGFP-expressing cells are distributed widely throughout the PC. eGFP-cells express vasopressin-neurophysin (VP-NP); the images show (**B**) fluorescence for eGFP, (**Bi**) immunoreactive VP-NP, and (**Bii**) overlaid images. **C,** vasopressin labelling in the PC of a wild-type rat. **Ci,** higher magnification of a cell. eGFP-cells co-express the vesicle glutamate transporter vGLUT-2 (white arrows) indicating that they use glutamate as a neurotransmitter; the images show (**D**) fluorescence for eGFP, (**Di**) vGLUT-2, (**Dii**) nuclear marker DAPI and (**Diii**) overlaid images.

Figure 2: Vasopressin neurons in the piriform cortex.

Immunohistochemistry for PC cell types Fluorescence immunohistochemistry showing that some eGFP-expressing cells (green) do co-express GABA, calcium binding proteins such as calbindin and calretinin, but not parvalbumin. There is no co-localisation with cholecystokinin (CCK) or vasoactive intestinal polypeptide (VIP). Whereas vasopressin receptor staining was abundant in the PC, only a very few eGFP-vasopressin cells were immunoreactive for V1a or V1b receptors.

Figure 3: Sex differences in the number of vasopressin cells

A, Saggital section from the rat atlas showing quadrants analysed to determine the number of eGFP-expressing cells. Quantification of the number of GFP-positive cells along the PC show no sex differences (**B**) along the quadrants and (**C**) in the total number. **D,** Analysis of the number of GFP-positive cells in the BNST show significant fewer GFP-positive cells in females.

Figure 4: Fos expression in the olfactory system after LOT stimulation

A-D, Quantification of Fos-positive cells and (**Ai-Di**) representative images with (**Aii-Dii**) enlargements from (**A**) mitral cell layer in the MOB, (**B**) mitral cell layer of the AOB, (**C**) dorsal area of the AON and (**D**) PC. **E,** Coronal section from brain atlas showing target for electrode placement and (**F**) photograph of brain section showing electrode tract in the LOT. Proportion of Fos-positive vasopressin cells in the (**G**) AON and (**H**) PC. Mean ± SEM, *P ≤ 0.05, **P ≤ 0.01.

Figure 5: Fos expression in the SON after LOT stimulation

A, representative image of a SON showing Fos expression in vasopressin co-labelled cells. Quantification of (**B**) Fos-positive cells per section of the SON and (**C**) proportion of Fos-positive vasopressin cells in response to LOT stimulation. Mean ± SEM, *P ≤ 0.05,

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