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Effects of lateral olfactory tract stimulation on Fos immunoreactivity in vasopressin neurons of the rat piriform cortex

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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

25 expressed in vasopressin neurons (eGFP-vasopressin), we show here a population of
26 vasopressin neurons in the PC. The vasopressin neurons are predominantly located in the
27 layer II of the PC and the majority co-express the excitatory transmitter glutamate.
28 Furthermore, there is no sex difference in the number of neurons expressing vasopressin.
29 Electrical stimulation of the lateral olfactory tract (LOT) leads to a significant increase in
30 the number of Fos-positive nuclei in the PC, MOB, AOB, dorsal AON, and supraoptic
31 nucleus (SON). However, there was only a significant increase in Fos expression in
32 vasopressin cells of the PC and SON. Thus functionally distinct populations of
33 vasopressin cells are implicated in olfactory processing at multiple stages of the olfactory
34 pathway.
35

36 **Abbreviations:** AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; BNST,
37 bed nucleus of the stria terminalis; MOB, main olfactory bulb; LOT, lateral olfactory
38 tract; PC, piriform cortex; SON, supraoptic nucleus
39

40 **Keywords**: anterior olfactory nucleus, main olfactory bulb, accessory olfactory bulb,
41 supraoptic nucleus
42

43 **Running title:** Vasopressin and the piriform cortex
44

45 **Competing Interests Statement:** The authors declare that there are no conflicts of
46 interest.
47

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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.

The neuropeptide vasopressin, produced in the hypothalamus and secreted from the pituitary, has a key role in electrolyte and fluid homeostasis, but recent interest in vasopressin has been dominated by its functions within the brain and, especially on its involvement in social recognition. Vasopressin is involved in the modulation of social recognition at the level of the olfactory bulbs (7, 8) and brain regions such as the lateral septum (9, 10). The neuropeptide also regulates social behaviours such as aggression (11, 12), pair-bonding (13, 14), and parental behaviour (15, 16) and has also been linked to human social behaviours in health (17-19) and during neurological disorders such as autism (20, 21).

Previously, we reported that the rat olfactory system contains several population of interneurons which express vasopressin. They are localized in the MOB, AOB and anterior AON (7, 22). Vasopressin V1a receptors are expressed in the MOB and AON (7, 22-26) and V1b receptors in the AON, olfactory tubercle and PC (22, 27) which suggests that these cells might be sensitive to their own signal. Blocking the actions of vasopressin in the MOB impairs the social recognition abilities of rats and vasopressin agonists and antagonists can modulate the processing of information by olfactory bulb neurons (7, 8). Furthermore, exposure of adult rats to a conspecific juvenile, but not to a heterospecific predator odour, increases early growth response protein 1 (Egr-1) expression in vasopressin neurons of the AON (22). These data suggest that vasopressin neurons in the olfactory system are involved in the coding of social odour information (28, 29).

Here we describe a population of vasopressin neurons distributed across the length of the PC. As previously, by utilizing a transgenic rat line in which an enhanced green fluorescent protein reporter gene is expressed specifically in vasopressin cells (eGFP-vasopressin) (30) we characterised these neurons based on a number of other

95 chemical markers and determined whether there are sex differences in the number of
96 vasopressin-expressing cells in the PC. Finally, to understand how the inputs to the
97 olfactory system (OS) influence vasopressin neurons in the different parts of the olfactory
98 system and the hypothalamus, we electrically stimulated the lateral olfactory tract (LOT)
99 and measured the expression of the immediate early gene c-*fos* by immunocytochemical
100 detection of Fos, the protein product of c-*fos*, in wild type and transgenic rats.
101
102 **MATERIALS AND METHODS**
103 *Ethical Approval*
104 Procedures conducted in the UK were approved by the local Ethics Committee
105 and the UK Home Office under the Animals Scientific Procedures Act 1986. Experiments
106 were performed on adult male and female wild-type Sprague-Dawley and transgenic rats
107 (250-450 g), housed under controlled conditions (12 h light: 12 h dark, 21°C) with free
108 access to food and water. Most of the immunohistochemistry was carried out on a
109 homozygous line of transgenic rats expressing a vasopressin-eGFP (enhanced green
110 fluorescent protein) fusion gene (30).
111
112 *LOT stimulation*
113 Rats were anaesthetised with an i.p. injection of sodium pentobarbital (60 mg/kg)
114 and the level of anaesthesia was monitored throughout the surgical procedure.
115 Supplementary doses were administered as necessary.
116 The rats were prepared for dorsal surgery, and holes were drilled over both the
117 left and right LOT. A concentric stainless steel electrode (100 µm tip diameter, SNEX-
118 100, Clark Electromedical Instrument, Kent, UK) was lowered into the LOT (below the

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

129 The electrode was then removed and placed in the LOT of the left hemisphere
130 for 10 min without any electrical stimulation. For the control group, the electrode was
131 placed to the both sides of the LOT for 10 min without stimulation. To mimic the
132 activation throughout the olfactory system by the input, we applied 10 min of 50 Hz
133 electrical stimulation to the LOT and analysed the expression of Fos. At 90 min after the
134 end of electrical stimulation, rats were terminally anesthetized and transcardially perfused
135 for tissue collection. The position of the stimulating electrodes in LOTs was verified
136 histologically (Fig. 4F).
137

138 *Tissue preparation*

139 Rats were terminally anesthetized (isoflurane inhalation then sodium
140 pentobarbital, 200 mg/kg body weight, i.p.) and transcardially perfused with a heparinised
141 (20 U/ml) 0.9% saline solution followed by paraformaldehyde (PFA) 4% in 0.1 m
142 phosphate buffer (PB). The brains were removed and immersed overnight in a solution

143 of 2% paraformaldehyde and 15% sucrose in 0.1 M PB at 4ºC. Then the brains were
144 placed in a solution of 30% sucrose in 0.1M PB and left for at least 72 h before they were
145 processed. The rat brains were cut using a freezing microtome and stored in a
146 cryoprotectant solution (30% ethylene glycol + 20% glycerol in 0.05 M sodium phosphate
147 buffer, pH 7.3) at 4°C until required.
148

149 *Immunocytochemistry*

150 Immunochemistry on free-floating sections was performed for both wild type
151 and transgenic rats. For wild-type rats, coronal sections were cut at 40 μm and washed in
152 PB + 0.2% Triton X-100 for 3 times to remove excess fixative/cryoprotectant. Sections
153 were then given a 5-min wash in PB before blocking endogenous peroxidase using the
154 methanol solution (PB + 20% v/v methanol + 0.3% w/v hydrogen peroxide). Again, the
155 sections were washed with PB+ 0.2% Triton X-100 three times for 5 min. To block non-
156 specific staining, sections were incubated for 30 min in a PB blocking buffer consisting
157 of 1% normal sheep serum + 0.3% Triton X-100. The sections were incubated in the c-
158 Fos antibody (Ab-2, Oncogene Sciences, Cambridge Bioscience, UK) at 1:1000 dilution
159 in PB blocking buffer for an optimum of 36 h at 4°C. After sections were washed in PB
160 + 0.2% Triton X-100 for three times, they were incubated for 60 min with Biotinylated-
161 anti-rabbit IgG (1:100, Vector Laboratories, Inc., Peterborough, UK) in PB+ 3% normal
162 goat serum+0.2% Triton X-100 at room temperature. Sections were next incubated for 60
163 min in ABC complex diluted as detailed by the manufacturer (Vectorstain elite ABC kit,
164 Vector Laboratories, Inc., Peterborough, UK). The sections were rinsed twice in PB +
165 0.2% Triton X-100, followed by 0.2M acetate buffer (pH 6.0) and visualized by
166 incubating the sections in the glucose oxidase-Ni DAB (3, 3'- diaminobenzidine) solution

167 (0.025% DAB + 2.5% Nickel II sulphate + 0.08% ammonium chloride + 0.4% β-D-
168 Glucose + 0.003% Glucose oxidase + 0.2M acetate buffer).

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

191 ascending concentrations of ethanol (70% and 90% for 5 min each then 95% and 2 x
192 100% for 10 min each and then Xylene 2 x 10 min), slides were cover slipped using DPX
193 mountant.

194 For immunohistochemistry involving exposure to a biotinylated secondary and
195 fluorescently tagged streptavidin, sections were blocked for endogenous biotin by
196 incubating them first in 0.01% avidin in 0.1 M PB for 30 min, washing and then
197 incubating in 0.001% biotin in 0.1 M PB for 30 min. After washing, sections were
198 incubated for 60 min in a blocking buffer consisting of 3–5% normal serum (matched to
199 the host of secondary animal) + 0.1% Triton X-100 diluted in 0.1 M PB. The sections
200 were incubated with primary antibodies (Table 1) diluted in the blocking buffer. The
201 primary antibodies were applied for 1–5 days at room temperature for the first day and
202 thereafter at 4°C. After washing in 0.1 M PB, sections were incubated for 60 min with the
203 appropriate secondary antibody (Table 2) and then washed in 0.1 M PB. Sections exposed
204 to biotinylated secondary antibodies were then incubated for 60 min with fluorescently
205 labelled streptavidin conjugate (1:500). Both secondary antibodies and fluorescently
206 labelled streptavidin were diluted in 0.1 M PB + 0.03% Tween20. After further washing,
207 sections were incubated in 4,6-diamidino-2-phenylindole (DAPI, 1:33 000, Life
208 Technologies Ltd, UK) for 5 min at room temperature, washed and coverslipped using
209 Permafluor Aqueous Mounting Medium (Thermofisher scientific, Waltham, MA, USA).
210 No fluorescent labelling was detected when primary antibodies were omitted or when the
211 primary antibodies (Table 2) were incubated with a fivefold (w/v) control immunogen
212 before being exposed to the tissue sections (the latter control was conducted whenever a
213 control peptide was available from the supplier of that primary antibody).
214

215 *Microscopy*

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

225

226 *Cell count*

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

235

236 *Quantification of Fos positive cells*

237 The number of Fos positive cells was quantified in a number of brain regions,
238 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).

263

264 *Statistics*

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

268

269 **RESULTS**

270 *Characterization of eGFP-vasopressin neurons in the PC*

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

280 Most of the vasopressin-immunoreactive cells were localised in layer II of the
281 PC, however a few were also seen in layer Ib and layer III (Fig. 1B); 63% of these cells
282 co-expressed glutamate (140 of 221 examined eGFP cells, Fig. 1D) and 20% co-
283 expressed GABA (56 of 257, Fig. 2). In very few cells, eGFP was co-localised with
284 calbindin (10.5%, 21 of 200) and calretinin (0.7%, 2 of 287), but not parvalbumin (0 of
285 323, Fig. 2), which label known subpopulations of PC neurons (36-38). There was no co-

286 localisation with cholecystokinin (CCK, 0 of 423) or vasoactive intestinal polypeptide
287 (VIP, 0 of 323) which have been described in neurons of the PC (Fig. 2) (36, 39).
288 Vasopressin receptor staining was abundant in the PC, but very few eGFP-
289 vasopressin cells were immunoreactive for V1a (6%, 22 of 365) and V1b receptors (1.8%,
290 3 of 163, Fig. 2).
291
292 *Sex differences*
293 To determine whether there are sex differences in the number of vasopressin
294 expressing cells in the PC as described for other areas harbouring vasopressin cells (33,
295 40), sagittal sections from eGFP-AVP transgenic rats were immunostained for GFP.
296 There was no significant difference between male and female rats in the number of
297 immunoreactive vasopressin cells in the PC (males 533 ± 71, females 589 ± 127; P=0.71,
298 Students t-test, n =6/group, Fig. 3B,C). By contrast, as expected, in the BNST there were
299 fewer immunoreactive vasopressin cells in female rats than in male rats (females 105 ±
300 15, males 204 ± 31, n = 4/group, P = 0.04; Fig. 3D).
301
302 *Fos induction after LOT stimulation*
303 Initially we studied the effects of electrical stimulation of the LOT on Fos
304 induction in the MOB mitral cell layer in coronal sections from wild-type SD rats. The
305 number of Fos-positive neurons was higher in the mitral cell layer ipsilateral to LOT
306 stimulation compared to the controls (61.6 ± 13.1 vs 20.7 ± 3.7 nuclei/section, p=0.02).
307 There was no significant difference in Fos expression in the mitral cell layer on the
308 contralateral side (contralateral stimulated, 11.0 ± 5.8 cells/section vs control, 17.2 ± 2.5
309 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

334 AON, but this did not reach statistical significance (control-ipsilateral of ventral vs dorsal,
335 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
336 bregma).
337 In the *dorsal* AON, LOT stimulation induced a significant increase in the number
338 of Fos-positive nuclei in the ipsilateral side compared to the control group (5.7 ± 1.4
339 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,
340 there was no significant difference in the levels of Fos expression between the control
341 and LOT stimulated group on either side (stimulated ipsilateral, 8.4 ± 3.6 cells/10^4 μm^2
342 vs control ipsilateral, 11.8 ± 4.7 cells/10^4 μm^2, p = 0.53).
343 There is a large number of vasopressin-expressing cells in the AON, but there
344 was no significant change in the proportion of vasopressin cells expressing Fos in
345 response to LOT stimulation in both groups ipsi- and contralateral (*dorsal*, control vs
346 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)
347 and contralateral (dorsal, control vs stimulated, 2.0 ± 1.4 vs 1.2 ± 0.4 % , p=0.90 , ventral,
348 10.0 ± 1.8 vs 9.8 ± 1.7 %, p=0.72, Fig.4G).
349
350 *Piriform cortex*
351 The PC runs in a band from the rhinal fissure to the ventral surface of the brain
352 following the curvature of the lateral wall. LOT stimulation induced a striking increase
353 in Fos expression in the PC (Fig. 4D). Following LOT stimulation 253 ± 61 cells/section
354 expressed Fos in the ipsilateral side, compared to 59 ± 12 cells/section in control rats (p
355 = 0.042). LOT stimulation also significantly increased the number of Fos-positive nuclei
356 in the contralateral side indicating a bilateral connection between the two hemispheres of

357 the olfactory system (stimulated contralateral, 252.2 ± 80.9 cells/section vs control
358 ipsilateral 58.8 ± 12.3 cells/section, p = 0.029, Fig. 4D).
359 There was also a significant increase in the proportion of Fos-positive eGFP cells
360 in the PC in the electrically stimulated ipsilateral side compare to control (stimulated
361 ipsilateral 37 ± 7%, vs control ipsilateral 6.0 ± 0.8%, p = 0.02, Fig. 4H).
362
363 *Supraoptic Nucleus*
364 Connectivity between the SON and the olfactory system has been described
365 previously (41-44). Therefore, we also analysed Fos induction after electrical stimulation
366 of the LOT in the SON (Fig. 5A). There was a significant difference between the control
367 and the electrically stimulated group in both hemispheres (stimulated ipsilateral 10.0 ±
368 $1.3/10^4$ μm^2 vs control ipsilateral, $2.3 \pm 0.7/10^4$ μm^2, p = 0.02, stimulated contralateral,
369 6.9 ± 0.8 $/10^4$ μm^2 vs control ipsilateral, $2.3 \pm 0.7/10^4$ μm^2, p = 0.03, Fig. 5B).
370 There was a significant increase in the proportion of Fos-positive eGFP cells in the
371 LOT stimulated ipsilateral and contralateral side compare to control (stimulated
372 ipsilateral 63.7 ± 5.8%, stimulated contralateral 54.5 ± 4.8% vs control ipsilateral 18.7 ±
373 6.7%, p = 0.02, 0.03, Fig. 5C).
374
375 **DISCUSSION**
376 Here we show a large number of cells expressing vasopressin in the PC. The PC
377 has been characterised both electrophysiologically and immunocytochemically (45-47)
378 and comprises a sparsely populated superficial layer I, a main input layer II containing
379 the densely-packed somata of glutamatergic principal neurons, and a deep layer III
380 containing principal neurons at lower density (48). Most of the vasopressin cells in the

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

387 We first discovered these cells in our transgenic rat strain in which eGFP is
388 expressed under the control of the vasopressin promoter. These rats have been extensively
389 used by us and others to study vasopressin-expressing neurons in distinct brain regions
390 (7, 22, 32, 49, 50). The vasopressin-eGFP transgene encodes a modified vasopressin
391 precursor with eGFP fused in-frame at the C terminus ((30) and D. Murphy, personal
392 communication). The signal peptide, vasopressin and neurophysin portions of the
393 precursor are intact, and may be expressed from the transgene, thus the vasopressin-
394 associated neurophysin in eGFP rats may reflect either endogenous expression or
395 transgene-driven expression. We therefore confirmed the expression of vasopressin in PC
396 cells in wild-type rats using antibodies against vasopressin-neurophysin.

397 Here we confirmed the presence of vasopressin receptor expression in the PC
398 (22, 27), however the receptors are not on the vasopressin cells themselves, indicating
399 that the neuropeptide is not involved in autoregulation as it is in the hypothalamus (51,
400 52). The PC is part of a network involved in the processing of olfactory cues used for
401 social communication (53-55) integrating odour features into odour objects (56). We and
402 others have previously shown that vasopressin is involved in the modulation of social
403 recognition at the level of the OB (7, 8) and the AON (22), suggesting that vasopressin
404 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.

452 We observed a significant increase in Fos expression in the contralateral side of the
453 PC following stimulation. The cortical feedback projection from PC to olfactory bulb is
454 complex and direct bilateral connections between PC have not yet been shown. However,
455 an ipsilateral projection from the anterior PC to the pars lateralis of AON has been
456 demonstrated (67) and thus the information transfer may occur to the contralateral PC
457 through the AON (68).
458
459 **Fos labelling in the SON**
460 In our experiments, LOT stimulation increased Fos expression in the SON.
461 Connectivity between the SON and the olfactory system has been described previously
462 (41-44, 69). Labelling studies have shown a monosynaptic pathway between the olfactory
463 bulb and the SON (42, 43, 70) and connections between olfactory sensory neurons and
464 the vasopressin neurons in the PVN and SON (44). In addition, Hatton and Yang
465 performed electrophysiological recordings *in vitro* and upon electrical stimulation of the
466 LOT they found that cells within the SON responded with short latency excitatory
467 responses supportive of a direct pathway from the olfactory bulb to the SON (41). Recent
468 studies indicate a role for oxytocin in the connections between the olfactory bulb and
469 PVN in the context of social interaction (57, 60). However, besides studies showing
470 anatomical connectivity between olfactory bulb and SON and PVN, the interplay between
471 these connections and the role of vasopressin needs to be determined in more detail.
472
473 Taken together, populations of vasopressin neurons in different areas of the rat olfactory
474 system, including the MOB, AOB and the AON have been shown to be involved in the
475 coding of social odour information (7, 22). Here we describe an additional population of

476 vasopressin cells in the PC. Together these functionally distinct populations of
477 vasopressin cells in different parts of the olfactory system suggest that vasopressin
478 signalling is involved in information processing at multiple levels of the olfactory
479 pathway.
480
481 FIGURE LEGENDS
482 **Figure 1: Vasopressin neurons in the piriform cortex.**
483 **A,** Coronal section from rat atlas indicating area of piriform cortex (PC) where images in
484 (**Aii, Aiii**) have been taken from, showing that eGFP-expressing cells are distributed
485 widely throughout the PC. eGFP-cells express vasopressin-neurophysin (VP-NP); the
486 images show (**B**) fluorescence for eGFP, (**Bi**) immunoreactive VP-NP, and (**Bii**) overlaid
487 images. **C,** vasopressin labelling in the PC of a wild-type rat. **Ci,** higher magnification of
488 a cell. eGFP-cells co-express the vesicle glutamate transporter vGLUT-2 (white arrows)
489 indicating that they use glutamate as a neurotransmitter; the images show (**D**)
490 fluorescence for eGFP, (**Di**) vGLUT-2, (**Dii**) nuclear marker DAPI and (**Diii**) overlaid
491 images.
492
493 **Figure 2: Vasopressin neurons in the piriform cortex.**
494 Immunohistochemistry for PC cell types Fluorescence immunohistochemistry showing
495 that some eGFP-expressing cells (green) do co-express GABA, calcium binding proteins
496 such as calbindin and calretinin, but not parvalbumin. There is no co-localisation with
497 cholecystokinin (CCK) or vasoactive intestinal polypeptide (VIP). Whereas vasopressin
498 receptor staining was abundant in the PC, only a very few eGFP-vasopressin cells were
499 immunoreactive for V1a or V1b receptors.

500

501 **Figure 3: Sex differences in the number of vasopressin cells**

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

507

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

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

515

516 **Figure 5: Fos expression in the SON after LOT stimulation**

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

520

521

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