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Effects of α-Melanocyte-Stimulating Hormone on Magnocellular Oxytocin Neurones and their Activation at Intromission in Male Rats

C. Caquineau,* G. Leng,* X. M. M. Guan,† M. Jiang,† L. Van der Ploeg† and A. J. Douglas*

*Centre for Integrative Physiology, University of Edinburgh, Edinburgh, UK.

†Departments of Medical Chemistry and Obesity Research, Merck Research Laboratories, Rahway, NJ, USA.*

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*Correspondence to:
Dr Céline Caquineau, Centre for Integrative Physiology, University of Edinburgh, Edinburgh EH8 9XD, UK
(e-mail: c.caquineau@ed.ac.uk).*

The peptides α-melanocyte-stimulating hormone (α-MSH) and oxytocin have very similar effects on several behaviours, including male sexual behaviour. Both induce penile erection and enhance copulatory behaviour when given centrally, suggesting that their central actions are not independent. Here, we used intromission as a physiological stimulus to investigate whether some central effects of α-MSH during male sexual behaviour are mediated by oxytocin neurones. We used the expression of the immediate-early gene product Fos to investigate oxytocin neurone activation at intromission and after intracerebroventricular (i.c.v.) administration of α-MSH (1 μg/5 μl) and studied the effects of i.c.v. administration of a MC4 receptor antagonist on Fos expression and on the latency of male rats to exhibit sexual behaviour in the presence of a receptive female. In rats that showed intromission, Fos was expressed in magnocellular oxytocin neurones in both the paraventricular nucleus (PVN) and the supraoptic nucleus (SON), but there was no significant activation of parvocellular oxytocin neurones of the PVN. Similarly, α-MSH increased Fos expression in magnocellular oxytocin neurones but had little or no effect in parvocellular oxytocin neurones. In male rats that achieved intromission, central injection of a MC4 receptor antagonist significantly attenuated the increase in Fos expression in magnocellular oxytocin neurones in both the PVN and the SON and increased mount and intromission latencies compared to vehicle-injected controls. Together, the results indicate that magnocellular oxytocin neurones are involved in the central regulation of male sexual behaviour, and that some of the central effects of α-MSH are likely to be mediated by magnocellular oxytocin neurones.

Key words: Fos, supraoptic nucleus, paraventricular nucleus, copulation, intromission, hypothalamus.

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α-Melanocyte-stimulating hormone (α-MSH) is synthesised in the hypothalamus by neurones of the arcuate nucleus that project centrally. The targets of this projection include the oxytocin neurones in the paraventricular and supraoptic nuclei (PVN and SON, respectively) (1, 2). α-MSH is involved in several behaviours, including feeding, the stretching-yawning reflex, pain perception, and male and female sexual behaviour. When given centrally, the effects of α-MSH are remarkably similar to those of oxytocin. Both α-MSH and oxytocin reduce food intake (3, 4) induce grooming and yawning (5, 6), are anxiolytic (7) and stimulate sexual behaviour. In females, they enhance female sexual receptivity and performance (8) and, in males, they induce penile erection and stimulate sexual performance (9, 10). For both peptides, these effects are inhibited by opioids (11) and NOS inhibitors (11, 12), suggesting that their central actions are not independent. The central effects of α-MSH on male sexual behaviour are thought to be mediated via MC3 receptors (6) or MC4 receptors (10, 13). MC4 receptor mRNA is expressed at high levels in the SON and in the PVN (14, 15).

Magnocellular oxytocin neurones of the SON and PVN have just a single axon that projects to the neurohypophysis, and few collateral branches of these axons terminate within the brain. Accordingly, the source of central oxytocin mediating behaviours has generally been assumed to be centrally-projecting parvocellular oxytocin neurones of the PVN. However, the dendrites of magnocellular neurones are by far the largest source of central oxytocin, and very large amounts of oxytocin are released from them in response to

many physiological and chemical stimuli *in vivo* (16). In particular, Sabatier *et al.* (17) showed that α-MSH stimulates dendritic release of oxytocin via an intracellular calcium-dependent mechanism; these effects can be blocked by an MC4 receptor antagonist. The actions of α-MSH result in Fos expression in magnocellular neurones in both the SON and the PVN (3, 17, 18). Oxytocin neurones, including magnocellular neurones, also express Fos during male sexual behaviour (19, 20), and *ex copula* electrical stimulation of the dorsal penile nerve increases the activity of oxytocin neurones in the SON (21) and PVN (22). In the present study, we used intromission as a stimulus to investigate whether some of the central effects of α-MSH on sexual behaviour might be mediated by oxytocin neurones via MC4 receptors in male rats.

Materials and methods

All animal experiments were performed under project and personal licenses awarded by the UK Home Office, and strictly in accordance with Home Office guidelines. Adult male (weighing 350–450 g) and female (weighing 250 g) Sprague-Dawley rats with food and water *ad lib* were housed under a 12 : 12 h light/dark cycle at an average room temperature of 22 °C. For behavioural studies, rats were placed in a reversed light cycle, (lights off 07.00 h) for 2 weeks before and during the experiments.

Induction of female receptivity

Female rats were ovariectomised bilaterally under halothane inhalation anaesthesia (3% halothane in 1200 ml/min both O_2 and nitrous oxide) and brought into oestrus by an s.c. injection of 3-β-estradiol monobenzoate (30 μg in 300 μl; ICN Biomedicals Inc., Irvine, CA, USA) and progesterone (1 mg in 100 μl; Sigma, Poole, UK), respectively, 48 h and 6 h before the experiment (23). Their receptivity was tested directly before each experiment by pairing each female with a sexually-experienced male (male that had at least two previous sexual experiences); only females that responded with lordosis within 10 min were used.

Intracerebroventricular (i.c.v.) cannulation

Under brief halothane anaesthesia, male rats were fitted with an i.c.v. guide cannula (22-gauge, 4.5 mm long; Bilaney Consultants Ltd, Sevenoaks, UK) positioned at 0.6 mm posterior and 1.6 mm lateral to bregma (24). Rats were allowed at least 3 days to recover. The i.c.v. injection cannula (5.5 mm length), attached to a Hamilton syringe via fine tubing, was inserted into the guide cannula 1 h before the experiments to avoid any stress that might complicate interpretation of the data. After each experiment, microscopical location was used to check the i.c.v. injection site in 44-μm frozen brain sections. Only brains with a confirmed correct injection site were processed for immunocytochemistry.

Recording of mount and intromission latencies

Males were placed in the cage of a receptive female, and their behaviour was recorded for 15 min. As a measure of the rat's motivation and ability to copulate under different treatments, mount and intromission latencies were recorded. A 'mount' was recognised as when the male mounted the female from the rear and grasped her flanks with his front feet, eliciting lordosis. 'Intromission' was recognised when the male achieved vaginal penetration and dismounted (25).

Effect of i.c.v. injection of α-MSH on Fos expression in oxytocin neurones

Male rats were implanted with an i.c.v. cannula as described above. On the day of the experiment, they were given an i.c.v. injection of α-MSH (1 μg in 5 μl; Calbiochem-Novabiochem, Nottingham, UK) freshly dissolved in isotonic saline, or the equivalent volume of isotonic saline (controls). At 90 min after drug administration, the rats were killed with an overdose of sodium pentobarbitone (1 ml i.p.) and transcardially perfused with heparinised 0.9% physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The brains were removed, postfixed overnight, and cryoprotected in 30% sucrose, and stored at 20 °C before processing for immunocytochemistry.

Effect of intromission on Fos expression in oxytocin neurones

Five sexually-naïve and seven experienced male rats were used to investigate whether sexual experience has an effect on Fos expression during mating. The experienced rats had previously been placed in a mating cage with a female for two consecutive 4-day periods. The presence of a plug, reflecting successful copulation, was checked every morning. Only males with successful copulations were considered as 'experienced'. During the experiment, behaviour was recorded for 15 min after introducing a sexually receptive female rat. Controls were males placed in a novel environment; they received no sexual stimulation, nor any female cues. After 15 min, the male rat was returned to its home cage. At 90 min after intromission, rats were perfused transcardially and the brains were removed and processed as described above.

Effect of i.c.v. injection of MC4 antagonist on Fos expression in oxytocin neurones at intromission

Male rats were implanted with an i.c.v. cannula as above. To establish that they were physically able to mate, their sexual vigour was tested before the experiment, to ensure confidence in the subsequent analysis of effects of drug treatment on mount and intromission latencies. Only males that successfully mated within 15 min when placed with a receptive female were used for subsequent experiments. On the day of the experiment, 10 min before being placed in the cage of a receptive female (26), rats were given an i.c.v. injection of a selective MC4 receptor antagonist (MARLON-0004; Merck Laboratories, Rahway, NJ, USA; 5.5 μg in 2 μl) (27), or an equivalent volume of isotonic saline (controls). Their behaviour was recorded for 15 min, or until intromission, and mount and intromission latencies were measured. Males that showed no mounts within 15 min were considered to be 'non-intromitting' males. After 15 min, the male rat was returned to its home cage. At 95 min after being placed with a receptive female, the male rats were perfused transcardially and their brains were removed and processed as above.

Immunocytochemistry

Coronal sections of hypothalamus (44 μm) were cut on a freezing microtome, and standard immunocytochemistry was performed on floating sections using a polyclonal antibody raised in rabbit against the N-terminal aminoacids 4–17 of the protein product of human c-fos (Ab-2; Calbiochem-Novabiochem) diluted at 1 : 1000 in preincubation buffer containing 1% normal sheep serum. For double immunocytochemistry (Fig. 1), a polyclonal antibody raised in rabbit against oxytocin (Ab-1; Oncogene Science, San Diego, CA, USA) was used at 1 : 1000 in preincubation buffer containing

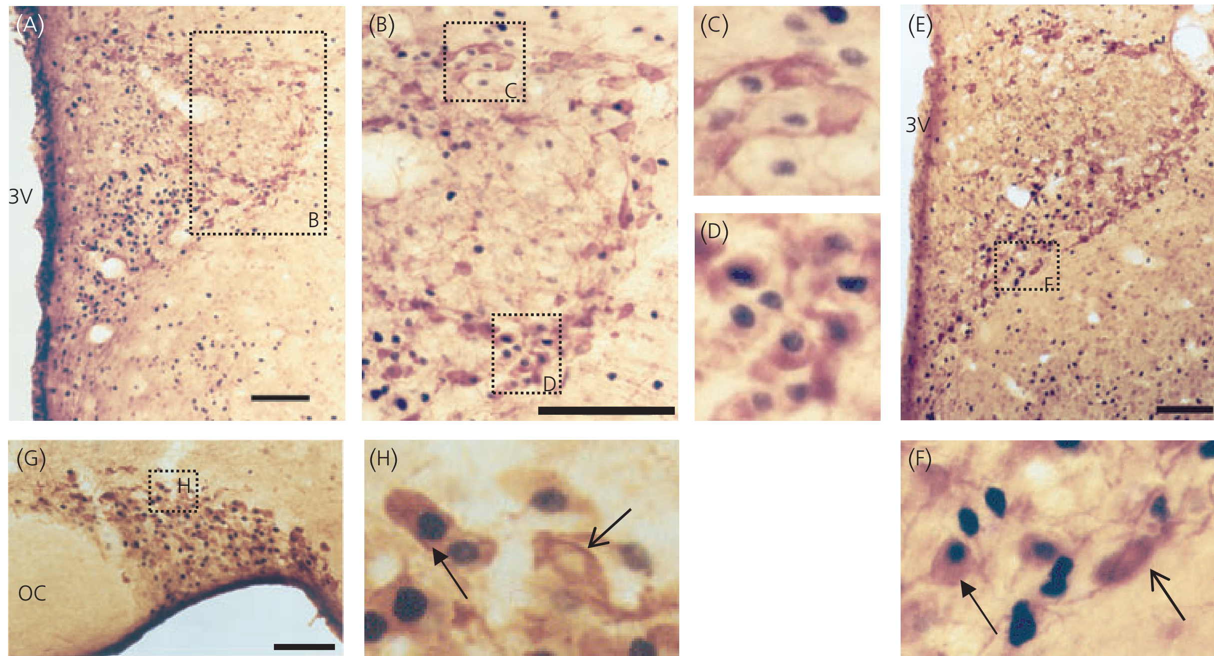

Fig. 1. Photomicrographs illustrating double labelling immunocytochemistry for Fos protein (black nucleus) in oxytocin neurones (brown cytoplasm) in the paraventricular nucleus (PVN) (A–F) and in the supraoptic nucleus (SON) (G,H). (B) Detail of the posterior magnocellular subdivision of the PVN from (A). (C) Detail of Fos-negative oxytocin neurones from (B). (D) Detail of Fos-positive oxytocin neurones from (B). (F) Detail of the ventral parvocellular subdivision of the PVN from (E). (H) Detail of Fos-positive oxytocin neurone (filled arrow) and Fos-negative oxytocin neurone (open arrow) from (G). 3V, Third ventricle; OC, Optic chiasm. Scale bar = 100 μm.

1% normal sheep serum. Antibody-antigen complexes were visualised using ABC methods with a Vector stain elite kit (Vector Laboratories, Bucks, UK) with nickel-intensified diaminobenzidine (Ni-DAB; for Fos, black nuclear label) or with DAB only (for oxytocin, brown cytoplasmic label) (28). Fos-positive neuronal nuclei were counted blind in the SON at the level of maximal cross-sectional area, in anterior, ventral, dorsal, and lateral posterior subdivisions of the parvocellular PVN (pPVN) and in the posterior magnocellular PVN (29). In general, three sections per rat were counted for the SON and two or three for each subdivision of the PVN.

Statistical analysis

Fos expression in different brain areas was compared between different groups of rats using a t-test where the experimental design involved comparing just two experimental treatments. For the experiments involving three groups (MC4 antagonist experiments), a one-way ANOVA was used followed by pairwise testing using parametric tests (Newman–Keuls) or their nonparametric equivalents (Dunn's method) where normality assumptions were violated. Mount and intromission latencies were compared between groups using an unpaired t-test. $P < 0.05$ was considered statistically different.

Results

Effect of α-MSH on Fos expression in the SON and in the PVN

As shown in previous studies (3, 18, 30), i.c.v. injection of α-MSH induced Fos expression in several areas of the central nervous system, including the PVN and SON. However, detailed analysis showed that there was no significant activation of Fos expression in any of the four parvocellular subdivisions of the PVN (Fig. 2A). By contrast, Fos expression in the magnocellular PVN was significantly higher in α-MSH-injected rats ($n = 5$) than in vehicle-injected rats ($n = 6$; 28 ± 3 versus 13 ± 2 cells/section; $P < 0.001$, Mann–Whitney rank sum test). This is consistent with our previous report of activation of magnocellular neurones in the SON (17).

After double-immunocytochemistry for Fos and oxytocin, a significant activation of oxytocin cells by α-MSH compared to vehicle controls was detected in just one parvocellular subdivision of the PVN, the ventral parvocellular PVN (Fig. 2B). In all other parvocellular subdivisions, there were no significant differences between vehicle-injected rats and α-MSH-injected rats. By contrast, a large and significant increase in Fos expression was measured in oxytocin neurones in the magnocellular PVN ($P < 0.001$, t-test; Figs 2B).

Effect of intromission on Fos expression in oxytocin neurones

The effect of intromission on Fos expression in oxytocin neurones was tested in all subdivisions of the PVN and in the SON. Controls received no sexual stimulation, or female cues; thus, the Fos expression in rats that achieved intromission and compared to controls reflects all prior components of sexual behaviour. Initially, separate groups of sexually-naive or experienced rats were used to investigate whether experience influenced the activation in the SON and PVN at intromission because previous studies had indicated

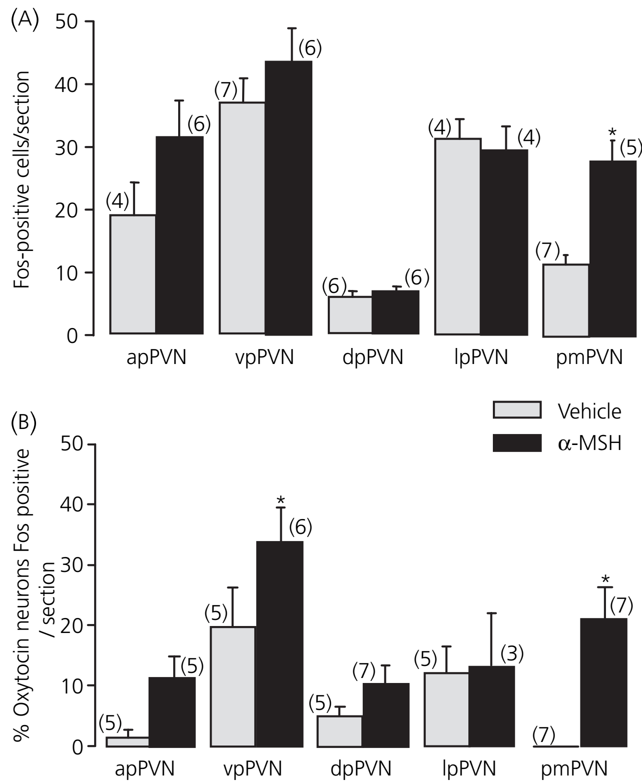

Fig. 2. Effect of i.c.v. injection of α-melanocyte-stimulating hormone (α-MSH) on Fos expression in oxytocin neurones in the paraventricular nucleus (PVN). Mean ± SEM percentage oxytocin neurones Fos-positive/section in the subdivisions of the PVN after i.c.v. injection of either vehicle (grey histograms) or α-MSH (black histograms). The number of rats per group is in parentheses. *P < 0.05 versus vehicle-treated group (unpaired t-test). apPVN, Anterior parvocellular PVN; vpPVN, ventral parvocellular PVN; dpPVN, dorsal parvocellular PVN; lpPVN, lateral posterior parvocellular PVN; pmPVN, posterior magnocellular PVN.

that sexual experience could affect Fos expression in response to female odours (31, 32). Intromission occurred at a shorter latency in sexually-experienced males than in sexually-naïve males (180 ± 18 s, n = 7 versus 324 ± 30 s, n = 5; P = 0.002, t-test), but there was no significant difference in Fos expression in oxytocin neurones in the PVN between sexually-naïve and experienced males (data not shown); thus, Fos data from these two groups were pooled. Compared to control rats, there was no significant activation in the parvocellular oxytocin neurones during male sexual behaviour in any subdivision of the pPVN (Table 1). However, after intromission, there was a small but significant increase in Fos expression in oxytocin neurones in the magnocellular PVN. No oxytocin cells expressed Fos in any of the control rats (n = 10), but there were 3 ± 1% Fos^+ oxytocin cells in rats that intromitted (n = 12; P = 0.02, t-test; Table 1).

In the SON, significantly more neurones expressed Fos in sexually-experienced rats that had mated than in control rats (8 ± 4 versus 2 ± 1 Fos^+ cells/section in sections stained for Fos alone, n = 5 rats per group; P = 0.02, t-test). On a set of adjacent sections, double immunocytochemistry was performed for Fos and oxytocin; Fos^+ cells

Table 1. Fos Expression in Oxytocin Neurones in Control and Mated Male Rats.

PVN subdivisions	Control	Mated
apPVN	0	2 ± 1
vpPVN	3 ± 2	8 ± 7
dpPVN	9 ± 5	10 ± 5
lpPVN	21 ± 10	12 ± 4
pmPVN	0	3 ± 1*

Mean ± SEM percentage Fos-positive oxytocin cells/section. apPVN, Anterior parvocellular paraventricular nucleus (PVN); vpPVN, ventral parvocellular PVN; dpPVN, dorsal parvocellular PVN; lpPVN, lateral posterior parvocellular PVN; pmPVN, posterior magnocellular PVN. *P = 0.02 versus control group (unpaired t-test).

were classed as positive or negative for oxytocin, or as 'not recognisable'. Of 239 identifiable neurones that expressed Fos in sections from rats that mated, 148 (62%) were oxytocin neurones (range 58–70%; mean per rat, 62 ± 2%).

MC4 antagonist delays the onset of mating

All sexually-capable male rats injected i.c.v. with vehicle exhibited female directed behaviours including mounts and intromission. By contrast, of eight sexually-capable rats injected with an MC4 antagonist, only five achieved intromission within 15 min. In the five antagonist-injected rats that did mate, behaviours were delayed compared to vehicle-injected rats (Fig. 3). These males displayed the first mount at a mean latency of 188 ± 55 s after pairing with a female versus 78 ± 24 s for vehicle-injected rats (P = 0.06, t-test). Intromission occurred after 274 ± 80 s in the five antagonist-injected rats that mated versus 127 ± 29 s in vehicle-injected rats (P = 0.05, t-test).

Effect of i.c.v. injection of MC4 antagonist on Fos expression in oxytocin neurones at intromission

For rats that achieved intromission, no significant differences in Fos expression in parvocellular oxytocin neurones were found in any subdivision of the pPVN between the antagonist-injected rats and the vehicle-injected rats (Fig. 4A); however, there was significantly less Fos expressed in magnocellular oxytocin neurones in the PVN of antagonist-injected rats than in vehicle-injected rats (P < 0.05; Fig. 4A). There was also significantly less Fos expression in magnocellular neurones in the SON in antagonist-injected rats than in vehicle-injected control rats (P < 0.05; Fig. 4B). In MC4 antagonist-injected rats that did not achieve intromission, there was no significant difference in the Fos expression in parvocellular oxytocin cells compared to those that achieved intromission (Fig. 4A). However, in both the magnocellular PVN and in the SON, significantly more oxytocin cells expressed Fos in rats that achieved intromission than in rats that did not (P < 0.05; Fig. 4A,B).

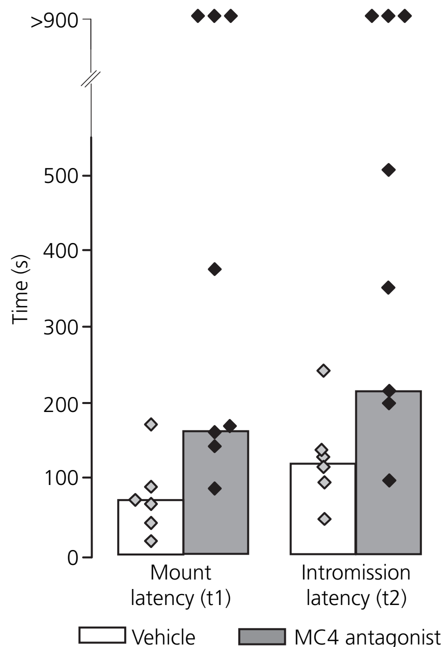

Fig. 3. Time to onset of copulation after i.c.v. injection of vehicle (white histograms) or MC4 receptor antagonist (grey histograms). The points show values for individual animals, the bars show medians of values for rats that achieved intromission. Three rats in the MC4 receptor antagonist-treated group did not mate (indicated by symbols at latency > 900 s). Mount latency, time (s) to first intensive mount; intromission latency, time (s) to intromission.

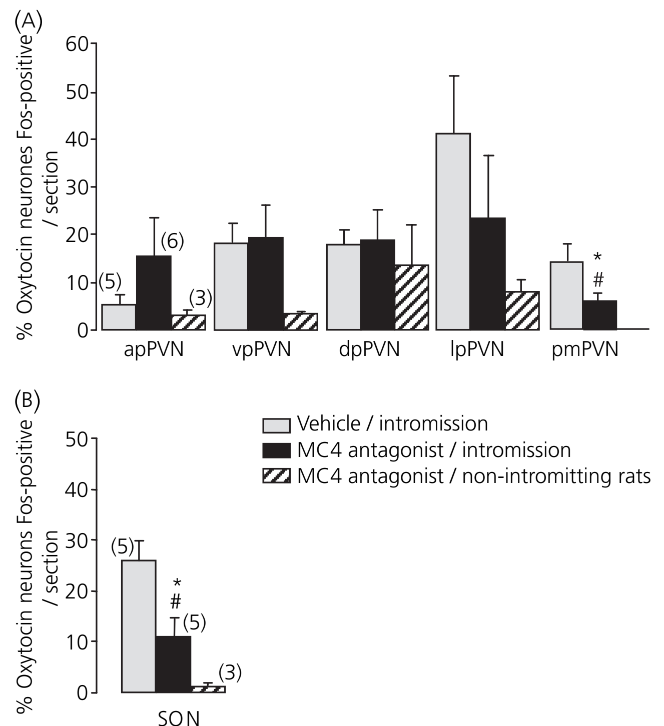

Fig. 4. Effect of i.c.v. injection of MC4 antagonist on Fos expression in oxytocin neurones in the paraventricular nucleus (PVN) and supraoptic nucleus (SON). (A) Mean ± SEM percentage oxytocin neurones Fos-positive/section in the PVN after i.c.v. injection of vehicle or MC4 antagonist in non-intromitting or intromitting rats. (B) Mean ± SEM percentage oxytocin neurones Fos-positive/section in the SON after i.c.v. injection of vehicle or MC4 antagonist in non-intromitting or intromitting rats. The number of rats per group is in parentheses. *$P < 0.05$ versus vehicle-treated group, #$P < 0.05$ versus MC4 antagonist/non-intromitting group (one-way ANOVA, Student–Newman–Keuls method or Dunn's method). apPVN, Anterior parvocellular PVN; vpPVN, ventral parvocellular PVN; dpPVN, dorsal parvocellular PVN; lpPVN, lateral posterior parvocellular PVN; pmPVN, posterior magnocellular PVN.

Discussion

In the present study, central injection of α-MSH activated magnocellular oxytocin neurones rather than parvocellular oxytocin neurones in conscious rats. It was also demonstrated that magnocellular oxytocin neurones of the SON and PVN were activated after intromission, and that central injection of a MC4 antagonist attenuated magnocellular oxytocin neuronal activity in the SON and PVN at intromission while delaying the onset of copulation, as shown by an increase in mount and intromission latencies.

Because parvocellular oxytocin neurones project centrally, whereas magnocellular oxytocin neurones project to the pituitary gland, it has been assumed that only parvocellular neurones are involved in oxytocin-dependent behaviours. Accordingly, central injection of α-MSH would be expected to activate parvocellular rather than magnocellular oxytocin neurones. However, in the PVN, α-MSH had little effect on parvocellular oxytocin neurones, but strongly activated magnocellular oxytocin neurones. We have previously shown that α-MSH acts directly on magnocellular oxytocin neurones to mobilise intracellular calcium, and local infusion of α-MSH via retrodialysis close to the SON significantly increases Fos expression in oxytocin neurones in the ipsilateral SON compared to the contralateral SON and compared to the SON of vehicle-treated rats (17). Together, these results suggest a direct action of α-MSH on magnocellular oxytocin neurones, rather than independent actions in the same target region.

It is also now shown that an increase in Fos expression in the SON and magnocellular PVN was evoked by a very brief encounter with a receptive female, up to intromission. Although significant, the increase in Fos expression in magnocellular oxytocin neurones was small, but small increases in expression appear to be typical of natural, physiological activation. Even at parturition, a very intense and extended physiological activation of oxytocin neurones, only a minority of them express Fos protein (33).

In 1994, Witt & Insel (19) studied the effect of male sexual behaviour on the activation of oxytocin neurones in the different subdivisions of the PVN by measuring Fos immunoreactivity in rats that achieved intromission only and rats that went on to ejaculation. They reported that, overall, the number of Fos-positive cells was increased in the PVN after intromission alone, but they found activation of oxytocin neurones only in only one parvocellular area, the lateral pPVN, compared to controls that had been exposed to female odours by being moved into cages vacated by receptive females. Their data are consistent with the present findings that the activity of most parvocellular oxytocin regions is not increased

in rats that achieved intromission within 15 min. These authors also found activation in magnocellular neurones in the PVN, but only in rats that had ejaculated, and not in rats that showed intromission alone. However, the level of Fos expression in their control group was high (23% of oxytocin cells in the magnocellular PVN of controls), and this might reflect activation by olfactory cues from the female even in the absence of intromission. In addition, the statistical power of their study to detect a difference was weak (only three rats per group were analysed).

In parvocellular subdivisions of the PVN, oxytocin neurones in the ventral parvocellular PVN (vpPVN) were activated after i.c.v. administration of α-MSH. However, the lack of significant increase in Fos expression in oxytocin neurones in the vpPVN during male sexual behaviour suggests that the increase in the vpPVN after α-MSH might not be correlated with the regulation of sexual behaviour. Oxytocin neurones from the vpPVN project to the brainstem, dorsal vagal complex and spinal cord (29). It is possible that the activation of oxytocin neurones in the vpPVN after α-MSH is related to the regulation of autonomic physiological functions such as cardiovascular functions, in which both α-MSH and oxytocin are involved.

Blocking central MC4 receptors delayed the onset of sexual behaviour, as reported previously by Martin *et al.* (10) and Van Der Ploeg *et al.* (13) who suggested that MC4 receptors participate in the modulation of erectile activity. Importantly, blocking the central actions of α-MSH also attenuated the activation of magnocellular oxytocin neurones in both the SON and PVN with no effect on Fos expression in parvocellular oxytocin neurones. We previously showed that α-MSH does not stimulate systemic oxytocin secretion, nor does it increase the electrical activity of magnocellular oxytocin neurones in the SON (17). However, MC4 agonists stimulate dendritic oxytocin release (17). Thus, some behavioural effects of α-MSH might be mediated by dendritic release of oxytocin from magnocellular neurones rather than from parvocellular neurones.

The role of dendritic release during sexual behaviour is unknown at present. During parturition and the milk ejection reflex, dendritic oxytocin release plays an essential role in oxytocin neurone discharge patterning to generate bursts of activity that produce a pulsatile pattern of peripheral oxytocin secretion (34), and dendritic oxytocin release might be important for later pulsatile oxytocin secretion during ejaculation. In addition, dendritic oxytocin may diffuse to other brain areas, given the very large amounts that are released from the dendrites and the long half-life of oxytocin in the brain (19 min in the CSF) (35). Thus, oxytocin might diffuse from its sites of release to interact with distant neuronal populations that densely express high-affinity oxytocin receptors (36). In conclusion, α-MSH may facilitate sexual behaviour in part via central oxytocin actions that may include short- and long-term effects, including by the ability to alter the functional connectivity of neuronal networks (37, 38).

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