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ORIGINAL ARTICLE

Intranasal Application of Vasopressin Fails to Elicit Changes in Brain Immediate Early Gene Expression, Neural Activity and Behavioural Performance of Rats

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Intranasal administration has been widely used to investigate the effects of the neuropeptides vasopressin and oxytocin on human behaviour and neurological disorders, although exactly what happens when these neuropeptides are administered intranasally is far from clear. In particular, it is not clear whether a physiological significant amount of peptide enters the brain to account for the observed effects. In the present study, we investigated whether the intranasal administration of vasopressin and oxytocin to rats induces the expression of the immediate-early gene product Fos in brain areas that are sensitive to centrally-administered peptide, whether it alters neuronal activity in the way that centrally-administered peptide does, and whether it affects behaviour in the ways that are expected from studies of centrally-administered peptide. We found that, whereas i.c.v. injection of very low doses of vasopressin or oxytocin increased Fos expression in several distinct brain regions, intranasal administration of large doses of the peptides had no significant effect. By contrast to the effects of vasopressin applied topically to the main olfactory bulb, we saw no changes in the electrical activity of olfactory bulb mitral cells after intranasal vasopressin administration. In addition, vasopressin given intranasally had no significant effects on social recognition or short-term recognition memory. Finally, intranasal infusions of vasopressin had no significant effects on the parameters monitored on the elevated plus maze, a rodent model of anxiety. Our data obtained in rats suggest that, after intranasal administration, significant amounts of vasopressin and oxytocin do not reach areas in the brain at levels sufficient to change immediate early gene expression, neural activity or behaviour in the ways described for central administration of the peptides.

Key words: vasopressin, oxytocin, olfactory bulb intranasal, mitral cells, c-Fos, blood pressure, behaviour

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In mammals, oxytocin and vasopressin have been implicated in the regulation of complex social behaviours, including attachment, social recognition and aggression, as well as anxiety-related behaviour (1). Accordingly, many recent studies have addressed the putative roles of these neuropeptides in human social behaviour (2). The peptides are now seen as potential targets for novel treatment approaches for human mental disorders characterised by social dysfunction, such as autism, social anxiety disorder, borderline personality disorder and schizophrenia (3,4).

To investigate whether the effects of these neuropeptides on behaviour are the result of a direct action on the central nervous system (CNS), the established experimental approach in laboratory rodents is to administer the substance either directly into brain tissue or into the cerebral ventricular system. In humans, different ways of administration must be used, although systemic administration is often problematical either because of poor penetration of the two neuropeptides across the blood–brain barrier, or because of their hormonal actions at peripheral targets (5). This has led

researchers to experiment with the intranasal administration of peptides, under the supposition that this route of administration might allow rapid access to adjacent brain regions at the same time as avoiding side effects.

Intranasal application of peptides has long been used as a way of administering them into the bloodstream (6). Intranasal administration of oxytocin was first used more than 50 years ago to evoke milk-let down in women during lactation (7), and it has been used in obstetrics to facilitate delivery for more than 80 years (8), exploiting the hormonal actions of oxytocin at the mammary gland and uterus, respectively. An extended form of arginine vasopressin, DDAVP, has a long history of use by intranasal administration for the treatment of diabetes insipidus in children (9,10).

Recent studies using a bewildering diversity of protocols (3,11) have reported many behavioural effects of the intranasal application of vasopressin and oxytocin in humans. However, there is no direct evidence that any oxytocin or vasopressin reaches the brain from any of the human studies. Only one study has measured oxytocin in cerebral spinal fluid (CSF) (12), where a very small rise after a large intranasal application was reported (a rise so small that it could reflect the release of endogenous oxytocin). Accordingly, it is important to determine whether any of the effects of the two intranasally-administered peptides are really mediated by activation of central vasopressin and/or oxytocin receptors, or by receptors in peripheral tissue, which then impacts on brain activity (13).

Although there is an extensive literature on the effects of vasopressin and oxytocin on a number of brain functions after i.c.v. injection in in rats and mice (14), to our knowledge, there is no published evidence of any such effects of intranasally-administered vasopressin and oxytocin. For example, i.c.v. injection or local brain application of either peptide triggers the expression of early immediate genes, including c-*fos*, in specific brain areas, especially those areas where specific receptors for vasopressin and/or oxytocin are expressed (15–20). Changes in the electrical activity of neurones in several of these brain regions have been reported by local and i.c.v. administration of the two peptides *in vivo* (21–24). Finally, central administration of oxytocin or vasopressin modulates several social behaviours, including social recognition, memory for peers, development of partner preference and bonding (14,25–27). One recent study in voles has reported some effects of intranasal oxytocin on social behaviour and anxiety (28).

The present experiments were designed to obtain an understanding of the central actions of vasopressin and oxytocin in rats after intranasal administration. We report a lack of effect of vasopressin and oxytocin after intranasal application in a variety of experimental designs, including studies on immediate early gene expression, blood pressure and heart rate, *in vivo* electrophysiology and behaviour testing.

Materials and methods

All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise stated.

Animals

Experiments were performed on adult male Sprague–Dawley rats (250–350 g), housed under a 12 : 12 h light/dark cycle at 21 °C, with free access to food and water. Experimental manipulations conducted in Germany were approved by the Committee on Animal Health and Care of the local governmental body and performed in strict compliance with the EEC recommendations for the care and the use of laboratory animals (86/609/CEE). 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 conducted in the USA were performed in accordance with institutional guidelines and with the approval of the Animal Care and Use Committees of the Wake Forest University. Only the minimum number of rats necessary to produce reliable scientific data was used.

Fos expression

Intranasal peptide administration

Rats were housed singly 3 days before the experiment and handled for 10–15 min each day to familiarise rats with the handler, and to minimise any stress-induced Fos response on the days on the experiment. On the morning of the experiment, the rats were briefly anaesthetised with isoflurane, and placed in a supine position, with the head supported at 45 ° to the body (29,30). Rats were given one of artificial cerebral spinal fluid (aCSF; 10 μl), vasopressin (1 μg dissolved in 10 μl of aCSF) or oxytocin (1 μg dissolved in 10 μl of aCSF) by slowly pipetting a 5-μl volume into each nostril when the rats were prone on their backs (n = 6 per treatment group). The doses of oxytocin and vasopressin given intranasally are equivalent to the total rat pituitary content of oxytocin and vasopressin and exceed (by 500-fold) the amounts needed to elicit effects by i.c.v. administration. After 5 min, rats were returned to their cages where they recovered from the anaesthetic until they were terminally anaesthetised for tissue fixation (see below).

Intracerebroventricular administration of peptide or vehicle

Rats were implanted with a left lateral ventricular cannula under isoflurane anaesthesia via a burr hole in the skull drilled 0.6 mm posterior to and 1.6 mm lateral to bregma. A guide cannula (4.5 mm long; Bilaney Consultants Ltd, Sevenoaks, UK) was secured in place using dental cement glued to two stainless steel screws driven into the skull. At this time, dummy caps were attached to the guide cannula to keep the guide cannula patent, and rats were singly housed. Rats were allowed 3 days to recover, and were gently handled for 10–15 min each day. On the day of experiment, each rat was briefly anaesthetised with isoflurane, the dummy cap removed and replaced with an injection cannula (5 mm long; Bilaney Consultants Ltd) connected to a Hamilton syringe with fine tubing, which had been pre-loaded with the peptide or vehicle solution. Each rat was injected with either 2 μl of aCSF or 2 ng of either vasopressin or oxytocin dissolved in 2 μl of aCSF (n = 6 per treatment group). The injection cannula was removed after 3 min and rats were returned to cages where they recovered from anaesthetic until terminally anaesthetised for tissue fixation (see below).

Tissue preparation

Previous studies with fluorescently-tagged peptide S showed fluorescent labelling in different brain regions at 15–30 min after intranasal administration (30), and the expression of Fos (the protein product of c-*fos*) is typically maximal by 90 min after an acute stimulus and plateaus for at least 1 h. Thus, 2 h after treatment (i.c.v. injection or intranasal treatment), rats were terminally anaesthetised for brain fixation by cardiac perfusion with 4%

paraformaldehyde solution. Rats were deeply anaesthetised (pentobarbital, 120 mg/100 g body weight by i.p. injection) and then perfused through the ascending aorta first with heparin (5000 U/ml; 300 ml) in 0.9% saline solution followed by 300 ml of a 4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution (pH 7.4). The brains were removed and immersed overnight in a solution of 0.2% paraformaldehyde and 15% sucrose in 0.1 M PB at 4 °C. The tissue was then placed in a solution of 30% sucrose in 0.1 M PB and left until the tissue had sunk (usually 48 h). The rat brains were snap-frozen and cut at 40 μm thickness using either a cryostat or freezing microtome. Sections were cut in 0.1 M PB and stored in a cryoprotectant solution (30% ethylene glycol + 20% glycerol in 0.05 M sodium phosphate buffer, pH 7.3) at −20 °C until used for immunohistochemistry. For each of the olfactory bulbs and hypothalami of each brain, every third section was collected together and processed by free-floating immunocytochemistry to detect Fos immunoreactivity, as described below.

Immunocytochemistry

After washing in 0.1 M PB to remove all cryoprotectant, sections were incubated for 20 min in 0.3% H_2O_2 in 0.1 M PB to quench endogenous peroxidase activity. Sections were washed at least four times for 10 min between each of the steps described below and, unless otherwise indicated, washed and incubated at room temperature. To block nonspecific interaction of secondary antibodies with the tissue, sections were then incubated for 60 min in a blocking buffer consisting of 3% normal horse serum + 1% bovine serum albumin + 0.2% Triton X-100 in 0.1 M PB. Sections were then incubated for 60 min at room temperature and then for 48 h at 4 °C with Fos primary antibody (PC38, polyclonal raised in rabbit, used at a dilution of 1 : 20 000 or 100 000; Calbiochem, San Diego, CA, USA), which was diluted in the blocking buffer. After extensive washing in 0.1 PB, the sections were incubated for 60 min with biotinylated-anti-rabbit immunoglobulin G (dilution 1 : 500, raised in horse; Vector Laboratories, Peterborough, UK) and washed again. Sections were next incubated for 60 min in ABC complex diluted in accordance with the manufacturer's instructions (Vectorstain Elite ABC kit; Vector Laboratories). After washing in 0.1 M PB, the sections were transferred to 0.1 M Tris solution (pH 7.4) for 5 min. Fos immunoreactivity was then visualised using a solution of 0.025% diaminobenzidine + 2.5% nickel II sulphate + 0.08% ammonium chloride + 0.015% H_2O_2 in 0.1 M Tris. A test section was first incubated in this solution to determine the optimal time to develop the diaminobenzidine (DAB) stain; in this case, it was found to be 6.5 min. After visualising, the sections were rapidly transferred back to 0.1 M Tris to stop further development of the DAB stain. After 5 min, sections were washed extensively in 0.1 M PB, and then finally in 0.05 M PB and mounted on gelatinised slides. After air-drying, the slide-mounted sections were counterstained (Nuclear Fast Red, for 5 min then washed in tap water for 3 min; Vector Laboratories), dehydrated in ascending concentrations of ethanol (70% and 80% for 5 min each, then 90% and 100% for 10 min each) and cover-slipped using DPX mountant (Sigma-Aldrich). No labelling was detected when primary antibodies were omitted (negative control).

Quantification of Fos-positive cells

Four investigators independently quantified the number of Fos-positive cells in a number of brain regions, including the main olfactory bulb, accessory olfactory bulb, anterior olfactory nucleus (each with a number of subregions), the piriform cortex, lateral septum (divided into dorsal and ventral), supraoptic nucleus, paraventricular nucleus, paraventricular thalamic nucleus, suprachiasmatic nucleus and amygdala. Another investigator repeated counting of selected regions to confirm consistency. All of the investigators were blinded to the treatments at the time of counting. Images of the regions were acquired using a Leica digital camera, controlled by Leica acquisition software, and attached to an upright Leica microscope and × 10 objective (Leica Microsystems, Wetzlar, Germany). Images from at least six regions from every rat in each treatment group were acquired. Using IMAGEJ (NIH, Bethesda, MD, USA), these images were converted to 8 bit, thresholded using the same parameters, and Fos-positive cells were counted using the Analyse Particles macro. The conditions for thresholding and for the macro were determined in part by comparing the count results with manually-counted images and ensuring that the counts made manually and via the software matched for 20 regions. Because the size of the olfactory areas and piriform cortex exceeded the field of view or differed widely in size in the sections, non-overlapping, multiple regions of interest (ROI) were used. The number of Fos-positive cells within each ROI were normalised by the surface area of that ROI to allow comparison. Thus, the number of Fos-positive cells in these regions are expressed as the mean ± SEM per 10^4 μm^2 (corresponding to an area of 100 × 100 μm). Because all of the other areas described above were encompassed within the field of view, the total number of Fos-positive cells was counted for each region and is expressed as the mean ± SEM per section.

In vivo electrophysiology

In urethane-anaesthetised Sprague–Dawley rats (250–300 g), extracellular recordings were made using glass microelectrodes filled with 0.9% NaCl (tip diameter approximately 1 μm). Recordings were made from single mitral cells in the main olfactory bulb (approximately 7 mm anterior to bregma, approximately 1.3 mm lateral) and identified antidromically (collision test) by electrical stimulation of the ipsilateral lateral olfactory tract (1.4 mm posterior to bregma, 3.2 mm lateral, 9.5 mm deep, latencies to antidromic activation in the range 1.0–5.8 ms (24,31). Vasopressin (4 μg in 4 μl saline) or saline alone was given intranasally via a microsyringe connected to a thin polyethylene tubing (PE10) inserted into the nostril ipsilateral to the recording side after a stable period of recording of basal electrical activity. Activity quotients (i.e. the proportion of time active relative to total time) were calculated as described previously (24).

Behaviour

Drug administration

Thirty minutes before being tested in each behavioural test, rats were lightly anaesthetised with isoflurane (Baxter, Deutschland GmbH, Unterschleißheim, Germany). For the social discrimination test, rats were given bilateral intranasal infusions (5 or 10 μl per nostril; of either aCSF or vasopressin diluted in aCSF (50 ng or 10 μg) using a micropipette. For the elevated plus maze test and open field test, animals received 0.1 μg in 10 μl (5 μl per nostril). After infusion, rats were returned to their home cages and their quick re-awakening was recorded (all signs of drowsiness and ataxia as a result of anaesthesia vanished within 5 min).

Social discrimination

Olfactory-cued, short-term social recognition memory was tested during the light phase between 09.00 h and 15.00 h using the social discrimination test. Singly-housed adult male rats were tested in their home cages (37 × 21 × 15 cm). Stimulus animals (juveniles, 22–26 days old of both sexes) were isolated and kept individually in a fresh cage with food and water *ad lib.*, 2.5 h before starting the test. The test consisted of two 4-min exposures of the juvenile to the adult. A mirror behind the experimental subjects' cage facilitated the observation of the animals. During the first exposure, the adult investigates a juvenile rat to acquire its olfactory signature (Juvenile 1; J1). J1 is then removed and returned to its cage. After an interval of either 30 or 120 min, J1 is re-exposed to the adult, together

with a second, novel juvenile (Juvenile 2; J2). The time that the adult spends investigating each juvenile (direct sniffing and/or licking at the surface of the body of the juvenile) is measured by a trained observer who is blind to the animal's treatment using a computer keyboard by pressing pre-set keys. This allows for easy detection of the investigatory behaviour, even in freely-moving animals. A significantly longer investigation duration of J2 versus J1 is taken as evidence for an intact recognition memory (32). The procedures, including the age and sex of the juveniles, as well as the critical criteria and the appropriate analysis and presentation of the results obtained, are described in detail elsewhere (32).

Elevated plus maze

The elevated plus maze is widely used to monitor anxiety-related behaviour (33). In our laboratory, this is made of black polyvinyl chloride and has two open and two closed arms (50 × 10 × 40 cm) interconnected by a central platform (10 × 10 cm), and is mounted 50 cm above the floor. The brightness was set at 30 lux. For testing, a rat is placed on the central platform of the apparatus facing a closed arm, and its behaviour is recorded on video camera (mounted to the ceiling of the test room) and monitored from an adjacent room for 7 min. The number of entries into open/closed arms, and the time spent in open/closed arms are measured. An entry is defined as placing both forepaws into the given compartment of the maze. The maze is cleaned after each trial.

Open field

General behavioural activity including anxiety-related behaviour (34) was monitored in the open field. The apparatus existed of a white square open field (80 × 80 cm) with high walls (height 25 cm). The field was divided into squares (10 × 10 cm) marked on the floor. Testing was carried out under uniform bright illumination (315 ± 40 lux). On the testing day, a given experimental subject was placed in the centre of the field and the behaviour was recorded for 10 min using a video camera located above the centre of the arena. The recorded digital videotapes were analysed offline by a trained observer who was unaware of the animal's treatment. The behaviours scored were: time spent in the (unprotected) inner part of the field, time spent investigating the field (sniffing), time spent running (fast locomotion), number of lines (marking the 10 × 10 cm squares on the floor of the field) crossed in both the inner and the outer part of the field; number of episodes spent rearing (posture sustained with the hind paws on the floor) and grooming (including washing or mouthing of forelimbs, hind paws, face, body and genitals). Before testing each rat, the open field was carefully wiped with an alcohol solution and dried.

Blood sampling

Male rats were anaesthetised with urethane (1.25 g/kg i.p.; Sigma-Aldrich) and the left femoral artery was cannulated. Blood samples (0.3 ml) were withdrawn into heparinised syringes, centrifuged and the plasma separated and stored at −20 °C for the subsequent vasopressin radioimmunoassay. The remaining blood cells were suspended in an equivalent volume of 0.9% sterile saline and returned to the rat via the cannula. Blood samples were taken 10 min before, and then 15 and 45 min after, intranasal vasopressin (1 μg in 10 μl of aCSF) or vehicle administration. The vasopressin content of samples was measured by a specific radioimmunoassay after extraction, as described previously (35).

Blood pressure monitoring

Rats were anaesthetised with urethane (1.2 g/kg i.p.), placed on a warmed surgical platform and the catheters filled with heparinised saline (50 U/mL) were inserted into the left femoral artery and vein for blood pressure measurement and drug administration, respectively. The arterial catheter was connected to a pressure transducer (model 156 PC 15GWL; Microswitch, Freeport, IL, USA), connected to TS430 blood pressure amplifier (Transonic Systems, Ithaca, NY, USA) and cardiovascular parameters were measured with a computerised data collection system (IOX; EMKA Technologies, Falls Church, VA, USA). Arterial blood pressure and heart rate were monitored for 20 min (5 min before and 15 min after drug administration) Vasopressin (0.2 or 20 μg/10 μl), oxytocin (0.2 or 20 μg/10 μl) or aCSF (10 μl) by pipetting 5 μl into each nostril while the rats were prone on their backs. Vasopressin (0.05 μg/100 μl) and oxytocin (10 μg/100 μl) were also given i.v.

Statistical analysis

Statistical analysis was performed using SIGMASTAT (Systat Software Inc., Richmond, CA, USA) or GRAPHPAD PRISM, version 4.03 (GraphPad Software, San Diego, CA, USA). Immunohistochemistry data were analysed by the Mann–Whitney rank sum test or Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's post-hoc tests (to compare anaesthetic control with intranasal vehicle and vasopressin). Student's t-tests were used otherwise (after verifying that the data tested did not deviate from normality assumptions). Arterial pressure and heart rate were averaged in 10-s blocks. Baseline values were taken 1 min before drug administration and peak responses were compared with that at baseline. Statistical analysis was conducted using a two-way repeated-measures ANOVA with factors of drug administration (before and peak after administration) and dose (saline, low and high dose of peptide). Behavioural data were tested using Student's t-test, either paired (investigation durations for J1 versus J2 for each treatment separately) or unpaired for all parameters monitored in the open field. All values are expressed as the mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Fos expression

Fos, the protein product of the immediate-early gene c-*fos*, is a widely used marker of neuronal activation and several studies have shown that i.c.v. injection of vasopressin or oxytocin triggers the expression of Fos in a variety of brain regions (15–19). Vasopressin given i.c.v. at a dose of 2 ng induced significant increases in Fos expression in many areas of the olfactory system and in several hypothalamic nuclei (Fig. 1 and Tables 1 and 2). As expected, the amygdala, paraventricular nucleus, supraoptic nucleus and the lateral septum all showed significant increases in Fos expression in response to i.c.v. injection of either peptide compared to i.c.v. vehicle. The suprachiasmatic nucleus, the paraventricular thalamic nucleus and the piriform cortex responded significantly only to vasopressin, indicating peptide specificity (Tables 1 and 2).

By contrast, there was no significant difference in any brain region after intranasal administration of vasopressin compared to intranasal administration of vehicle (Table 1), even though these used a dose that was 500-fold higher than the dose shown to be effective by i.c.v. administration.

However, application of both vehicle and vasopressin increased Fos expression in regions the olfactory system compared to anaesthetic control (Table 1). Thus, Fos expression in the olfactory bulb is sensitive to nasal disturbances (even under anaesthesia), although there was no specific response to intranasal vasopressin.

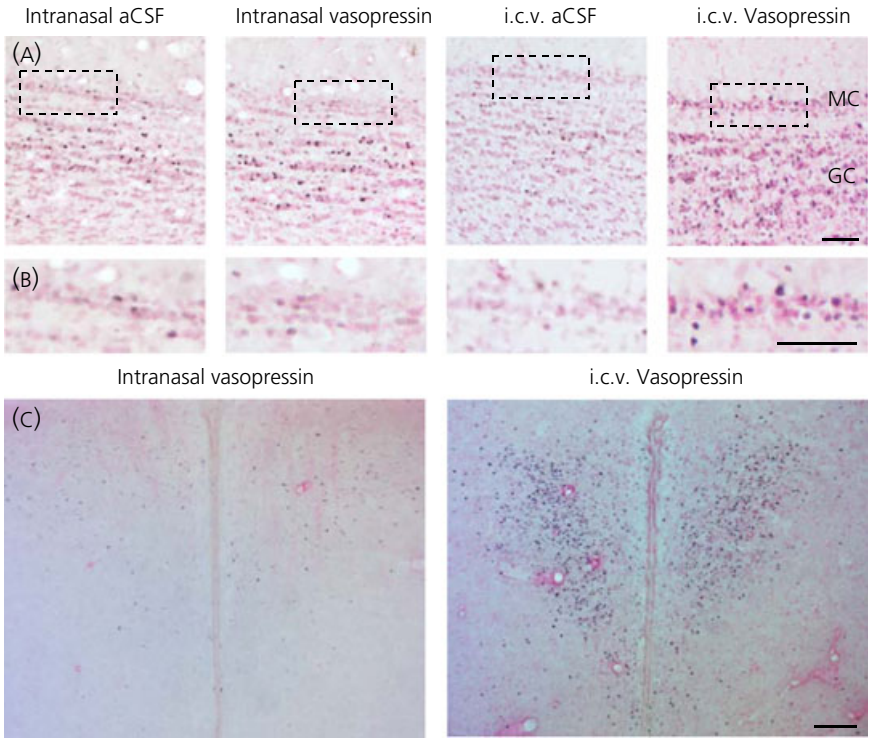

Fig. 1. Representative examples showing immunohistochemistry for the c-Fos protein in (A, B) the mitral and granule cell layer of the main olfactory bulb and (C) the paraventricular nucleus of the hypothalamus in response to intranasal or i.c.v. administration of vasopressin. aCSF, artificial cerebrospinal fluid; MC, mitral cell layer; GC, granule cell layer. Scale bars = 100 μm.

Electrophysiology

The main olfactory bulb contains receptors for vasopressin and oxytocin and intrinsic vasopressin cells that process olfactory signals relevant to social discrimination. We have previously shown that topical administration of vasopressin onto the exposed bulb dorsal to the recording site (in doses of 4 ng and 40 ng) significantly reduced the activity quotient of mitral cells (24).

We tested whether intranasally-administered vasopressin had a similar effect on mitral cell activity. Recordings in freely-breathing anaesthetised rats showed a patterned discharge comprising intermittent long bursts of action potentials, where the activity quotient measures the proportion of time active. However, intranasal administration of 1 μg vasopressin had no significant effect (activity quotient of 0.60 $\pm$ 0.08 before and 0.58 $\pm$ 0.06 after vasopressin, n = 6; 0.64 $\pm$ 0.13 before and 0.69 $\pm$ 0.09 after vehicle, n = 3) (Fig. 2).

Behaviour

Social discrimination

In the social discrimination test, the time that rats spend investigating a juvenile to which they have been previously exposed ('familiar' juvenile) is compared with the time they spend investigating a novel juvenile. Normal, untreated rats spend longer investigating novel juveniles than juveniles that they encountered 30 min previously, although juveniles encountered 120 min previously are investigated as much as novel juveniles (Fig. 3A) (32). Le Moal *et al.* (36) first showed that i.c.v. infusions of 0.5–2 ng of vasopressin improve short-term social recognition memory in rats (36), in line with the memory-improving effect of local treatment with vasopressin in this task (37,38). It has been shown previously that bilateral injection of 0.5 ng of vasopressin into the olfactory bulbs preserves social recognition responses (39), whereas blocking the actions of vasopressin in the olfactory bulbs (using antagonists or small interference RNA against the vasopressin V1a receptor, or local selective destruction of vasopressin cells with diphtheria toxin in transgenic rats) impairs the social recognition abilities of rats (24).

In the present study, we gave vasopressin at doses of 50 ng and 10 μg; these doses exceed the doses that have been shown to be effective centrally by 100–2000-fold. Neither Ringer's solution, nor vasopressin given intranasally had any significant effect on recognition performance, failing to affect either general social curiosity or to improve short-term recognition memory (Fig. 3B).

Elevated plus maze

In rats, vasopressin is implicated in the modulation of anxiety-related behaviour; for example, the administration of approximately 200 pg of vasopressin into the septum increases open-arm entries and open-arm duration in the elevated plus maze (40). We found no significant effects of intranasal infusions of vasopressin at a

Table 1. Number of Fos-Positive Nuclei in Areas of the Olfactory System (per 10^4 μm^2) and the Hypothalamus (per area) in Response to Intranasal and Intracerebroventricular Administered Vasopressin.

	Anaesthetic control	Intranasal aCSF	Intranasal vasopressin	i.c.v. aCSF	i.c.v. vasopressin
Main olfactory bulb					
Granule cells	0.4 ± 0.1	1.1 ± 0.1[a]	1.2 ± 0.1[a]	0.9 ± 0.2	18 ± 2*
Mitral cells	3.2 ± 0.5	10.2 ± 1.1[a]	9.6 ± 0.7[a]	1.2 ± 0.4	19.5 ± 2.7*
Epiform plexus	0.22 ± 0.06	0.29 ± 0.04[a]	0.3 ± 0.03[a]	0.4 ± 0.1	0.3 ± 0.1
Glomerular layer	1.1 ± 0.2	1.4 ± 0.2[a]	1.1 ± 0.1[a]	0.8 ± 0.01	0.99 ± 0.18
Accessory olfactory bulb					
Granule cells	0.09 ± 0.02	0.09 ± 0.04	0.08 ± 0.03	0.11 ± 0.04	0.08 ± 0.03
Mitral cells	0.82 ± 0.3	0.28 ± 0.07	0.25 ± 0.05	0.44 ± 0.28	1.2 ± 0.4*
Glomerular layer	0.5 ± 0.1	0.25 ± 0.09	0.2 ± 0.08	0.15 ± 0.06	0.12 ± 0.03
Anterior olfactory nucleus					
Pars interna (dorsal)	1.4 ± 0.4	11.5 ± 2.3	9.6 ± 1.7	0.8 ± 0.16	7.1 ± 1.1*
Pars interna (ventral)	0.1 ± 0.01	0.45 ± 0.05[a]	0.5 ± 0.05[a]	0.28 ± 0.06	4.8 ± 0.5*
Pars interna (lateral)	0.26 ± 0.06	3.54 ± 1.5[a]	2.7 ± 1.2[a]	0.13 ± 0.03	4.49 ± 0.45*
Pars interna (medial)	0.2 ± 0.04	1.1 ± 0.3[a]	0.75 ± 0.13[a]	0.06 ± 0.02	3.63 ± 0.26*
Piriform cortex	0.36 ± 0.04	0.38 ± 0.04	0.39 ± 0.04[a]	0.9 ± 0.14	2.7 ± 0.85*
Lateral septum					
Dorsal	4.3 ± 1.4	4.2 ± 0.6	3.1 ± 0.7	2.4 ± 0.4	21.7 ± 4.6*
Ventral	3.9 ± 1.1	5.5 ± 1	4.7 ± 0.7	4.5 ± 0.6	21.7 ± 3.8*
Supraoptic nucleus	1.4 ± 0.4	2.1 ± 0.6	2.1 ± 0.7	1.3 ± 0.3	4.1 ± 0.3*
Paraventricular nucleus	2.8 ± 0.4	2.1 ± 0.6	3 ± 0.6	1.44 ± 0.3	11.6 ± 1.3*
Paraventricular thalamic nucleus	2.4 ± 0.7	6.5 ± 1.5	5.6 ± 1.1	1.8 ± 0.5	51.4 ± 8.9*
Amygdala	6.2 ± 1.2	15.9 ± 2.4[a]	9 ± 1.2	14.1 ± 3.5	48.6 ± 8.7*
Suprachiasmatic nucleus	0.7 ± 0.2	1.3 ± 0.3	1.6 ± 0.7	0.7 ± 0.2	20.1 ± 2.2*

Data are the mean ± SEM. [a] Anaesthetic control compared to intranasal artificial cerebrospinal fluid (aCSF) and vasopressin. *$P < 0.05$ compared to i.c.v. vehicle.

Table 2. Number of Fos-Positive Nuclei in areas of the Olfactory System (per 10^4 μm^2) and the Hypothalamus (per area) in Response to Intranasal and Intracerebroventricular Administered Oxytocin.

	Intranasal aCSF	Intranasal oxytocin	i.c.v. aCSF	i.c.v. oxytocin
Main olfactory bulb				
Granule cells	0.5 ± 0.1	0.5 ± 0.1	1.3 ± 0.5	3.7 ± 1*
Mitral cells	1.4 ± 0.3	1.4 ± 0.4	2.7 ± 0.9	6.1 ± 2.2
Epiform plexus	0.2 ± 0.1	0.2 ± 0.1	0.26 ± 0.1	0.16 ± 0.04
Glomerular layer	0.01 ± 0.003	0.03 ± 0.01	0.008 ± 0.006	0.03 ± 0.01*
Accessory olfactory bulb				
Granule cells	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Mitral cells	0.2 ± 0.04	0.2 ± 0.03	0.2 ± 0.1	0.7 ± 0.2*
Glomerular layer	0.0 ± 0.0	0.01 ± 0.01	0.0 ± 0.0	0.01 ± 0.01
Anterior olfactory nucleus				
Pars interna (dorsal)	1.2 ± 0.2	1.9 ± 0.4	0.96 ± 0.17	1.1 ± 0.23*
Pars interna (ventral)	0.77 ± 0.14	0.87 ± 0.17	0.8 ± 0.2	2.1 ± 0.31*
Piriform cortex	0.27 ± 0.05	0.14 ± 0.03	0.19 ± 0.05	0.2 ± 0.04
Lateral septum				
Dorsal	1.5 ± 0.6	1.7 ± 0.5	2.4 ± 0.7	20.9 ± 3.1*
Ventral	2.6 ± 0.8	3.3 ± 0.9	2.3 ± 0.5	21 ± 2.7*
Supraoptic nucleus	0.1 ± 0.07	0.1 ± 0.04	2 ± 0.3	2.8 ± 0.3*
Paraventricular nucleus	4.4 ± 1.2	3.3 ± 0.7	1.4 ± 0.2	9.2 ± 0.8*
Paraventricular thalamic nucleus	0.6 ± 0.2	0.7 ± 0.2	2 ± 0.4	0.9 ± 0.2
Amygdala	1.5 ± 0.4	1.1 ± 0.3	0.9 ± 0.3	41.3 ± 5.5*
Suprachiasmatic nucleus	0.5 ± 0.2	0.5 ± 0.2	1.3 ± 0.3	0.8 ± 0.2

Data are the mean ± SEM. *$P < 0.05$ compared to i.c.v. artificial cerebrospinal fluid (aCSF).

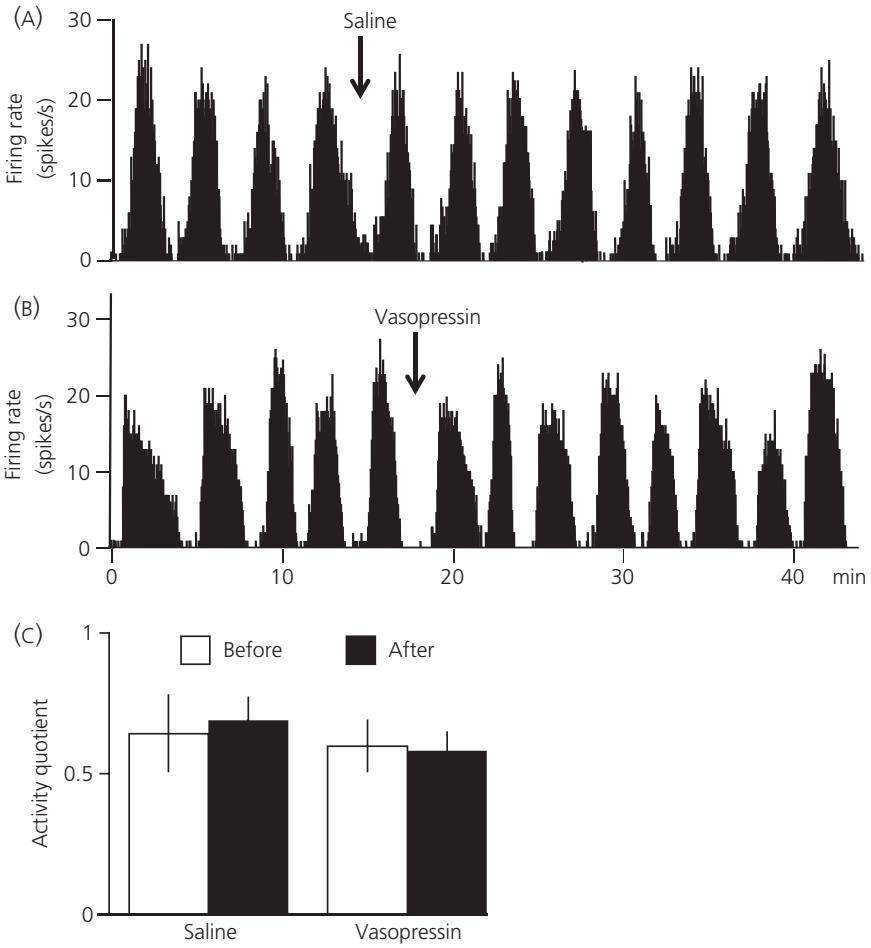

Fig. 2. Firing rate of identified mitral cells before and after intranasal administration of saline (A) or vasopressin (B). (C) The activity quotient (proportion of time active relative to total time) did not change in response to intranasal saline (n = 3) or vasopressin (n = 6) administration. Data are the mean ± SEM.

dose that was 5000-fold higher (0.1 μg) on any of the parameters monitored on the elevated plus maze (Fig. 3C).

Open field

A recent study reported that, in the open field test, female voles cross fewer lines after long-term treatment with intranasal oxytocin than voles treated with saline (28). Previous studies in rats have shown that i.c.v. injection of vasopressin does not affect open field behaviour (41). In the present study, we found no significant effect of intranasal infusions of vasopressin compared to vehicle on any of the parameters monitored on the open field test [time spent in the unprotected inner part of the field, time spent investigating the field (sniffing), time spend in running (fast locomotion), number of lines crossed in both the inner and the outer part of the field; number of episodes spent in rearing (posture sustained with the hind paws on the floor) and grooming; P-values between 0.17 and 0.99] (Fig. 3D).

Blood pressure

Injection of 1–10 ng of vasopressin into the lateral ventricles (i.c.v.) or various brain regions increases blood pressure and heart rate in rats, whereas i.c.v. injections of 0.3 μg of oxytocin reduce blood pressure, although the heart rate remains unchanged (42–44).

In the present study, intranasal administration of either 0.2 μg or 20 μg doses of vasopressin had a modest effect on arterial pressure and heart rate, although these effects were not significantly different from those that accompanied vehicle administration (Fig. 4). After intranasal administration of saline or vasopressin, there was a small increase in mean arterial pressure ($F_{1,4} = 25.4$, $P < 0.01$), although no effect of dose ($P < 0.18$) and no interaction between administration of test solutions and dose ($P < 0.3$), indicating that the increase was a nonspecific response to administering a solution intranasally. Intranasal administration of 0.2 μg of vasopressin increased arterial pressure by 8 ± 2 mmHg (from 88 ± 8 to 96 ± 7 mmHg), whereas a 100-fold higher dose (20 μg) similarly increased arterial pressure by 8 ± 1 mmHg (91 ± 6 to 99 ± 6 mmHg). Intranasal administration of saline increased mean arterial pressure by 5 ± 1 mmHg (89 ± 7 to 94 ± 7 mmHg). By contrast, i.v. administration of 0.05 μg of vasopressin produced a pronounced (52 ± 12 mmHg) and long lasting pressor response. Intranasally-administered saline or vasopressin also increased heart rate ($F_{1,4} = 7.8$, $P < 0.05$) but, again, this response was not dose-related ($P < 0.1$) and there was no

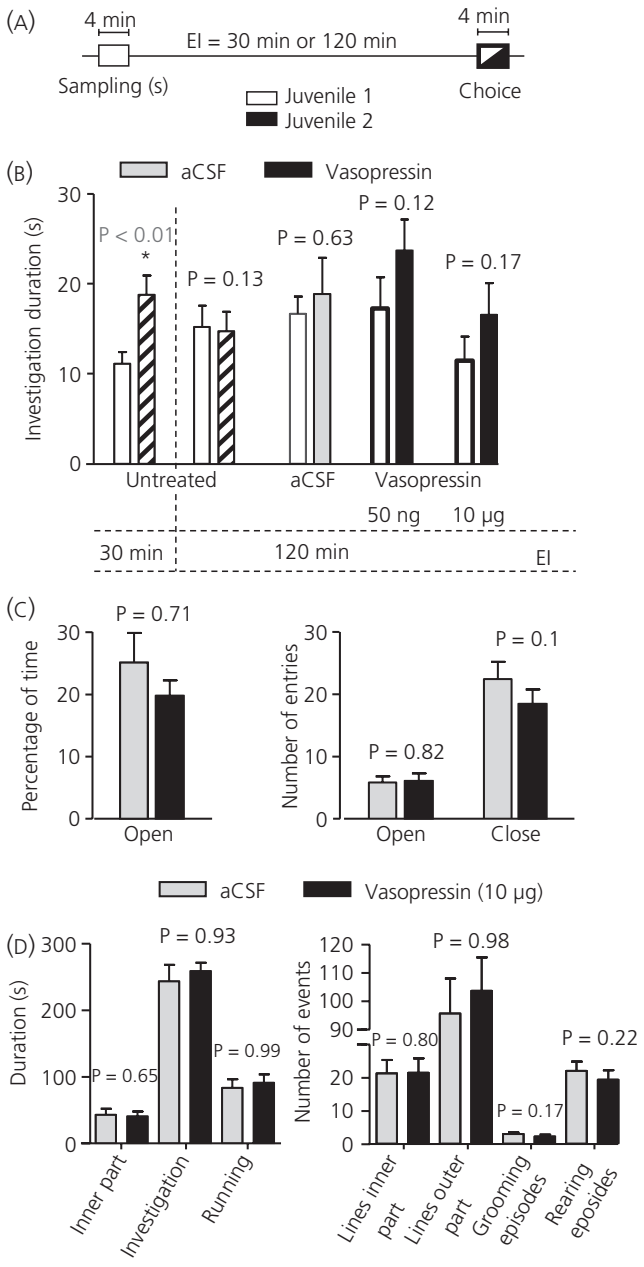

Fig. 3. (A) Experimental protocol used for testing the olfactory recognition memory as a function of the exposure interval (EI). During the sampling session (s), a given conspecific juvenile (Juvenile 1) was exposed for 4 min to the adult experimental subject. After an exposure interval of 30 min or 120 min, the same juvenile was re-exposed to the adult during a 4-min choice session together with a second, unfamiliar juvenile (Juvenile 2). (B) Investigation duration of adult male rats (control n = 12; vasopressin n = 11) during sampling and choice. None of the treatments was able to produce a discrimination at the exposure interval of 120 min between both juveniles, as indicated by the significantly longer investigation duration of Juvenile 2 versus Juvenile 1, and as observed at the exposure interval of 30 min under untreated conditions. No impact of intranasal infusion of vasopressin on anxiety-related behaviour, as measured in (C) the elevated plus maze or (D) open field test. Data are the mean ± SEM. *P = 0.01, paired Student's t-test. aCSF, artificial cerebrospinal fluid.

interaction between dose and the administration of test solution ($P < 0.3$). As expected, i.v. administration of vasopressin resulted in a pronounced bradycardia (346 ± 19 b.p.m. to 300 ± 17 b.p.m.).

Intranasal administration of oxytocin or a placebo also had only modest effects on arterial pressure ($F_{1,5} = 637$, $P < 0.001$) and heart rate (Fig. 4). Administration of 0.2 µg increased arterial pressure by 5 ± 1 mmHg (from 95 ± 17 mmHg to a peak of 101 ± 6 mmHg). Similarly, the higher dose of oxytocin had a similar effect, increasing arterial pressure by 7 ± 1 mm Hg from 93 ± 8 mmHg to 100 ± 7 mmHg. Again, there was no significant effect of dose ($P < 0.09$), nor was there a dose-by-administration interaction ($P < 0.8$), suggesting that any effect was the result of the administration of any solution via this route. In a similar manner, intranasal administration of solutions was associated with an increase in heart rate ($F_{1,5} = 11.2$, $P < 0.02$) but, again, there was no effect of dose ($P < 0.6$) and no interaction effect ($P < 0.4$). By contrast, i.v. administration of 10 µg oxytocin increased arterial pressure by 47 ± 9 mmHg (from 97 ± 8 mmHg to 144 ± 3 mmHg). As with vasopressin, this pressor response was associated with a bradycardia (355 ± 20 b.p.m. to 303 ± 17 b.p.m.). Intravenous administration of oxytocin can exert vasodilatory effects mediated through a nitric oxide mechanism (45), although, at high concentrations, oxytocin has pressor actions (46) through the stimulation of vasopressin receptors (47–49); at the dose given (10 µg), this was as expected (this dose was chosen to be 200-fold higher than the dose of vasopressin given i.v.).

Plasma sampling

There were no significant effects of intranasal infusions of vasopressin on plasma vasopressin concentrations at any sampling point (Fig. 4E) compared to vehicle.

Discussion

The present study was designed to unravel, in the laboratory rat, the intracerebral targets and physiological consequences of intranasally-delivered vasopressin and oxytocin, which were previously shown to be highly effective in modulating the behavioural response in humans.

When delivering doses of vasopressin or oxytocin this large into the nasal cavity in any study, careful consideration needs to be given with respect to exactly where it goes. Why it should ever be assumed that drugs delivered intranasally do not enter the bloodstream is not clear. Indeed, the intranasal application of peptides has long been used as a way of administering them into the bloodstream; deep inhalation of aerosols delivers substances into the lungs, where they can readily cross the permeable membranes of bronchial capillaries. As noted, an extended form of arginine vasopressin, DDAVP, has long been used intranasally for the treatment of diabetes insipidus in children (9,10); the effectiveness of which requires that the administered peptide reaches the kidneys. Intranasal administration of oxytocin was first used to evoke milk-let down in women during lactation (7) and it has been used in obstetrics to facilitate delivery (8), exploiting the actions of oxytocin at the

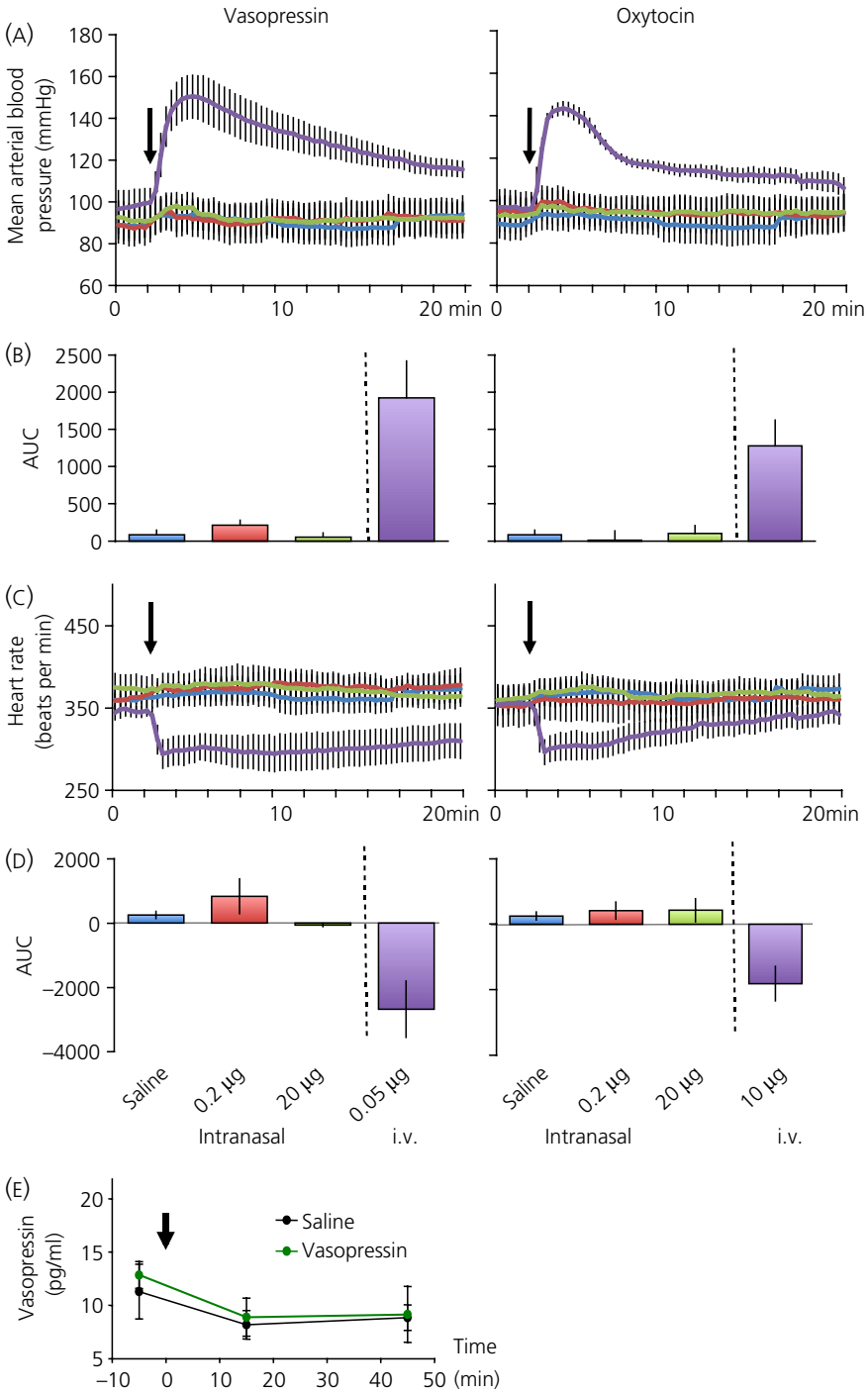

Fig. 4. Measurements of mean arterial blood pressure (A) and heart rate (C) in response to intranasal or i.c.v. administration of saline, vasopressin or oxytocin. (B, D) Area under the curve (AUC) for the treatments. (E) Vasopressin content in plasma samples before and after intranasal administration of vasopressin or saline (n = 6 each group). Data are the mean ± SEM.

mammary gland and uterus, respectively. In 1961, the first placebo-controlled clinical trial of intranasal oxytocin for milk let-down appeared (50), reporting that the administration of 4–5 IU (8–10 μg) of oxytocin intranasally to lactating mothers before each feeding session led to an increased weight gain of the babies. In 1971, a study of its use in 1800 patients in two community hospitals concluded that, when given as doses of 0.4–0.8 IU (0.8–1.6 μg) every 15–20 min, it was safe and effective in augmenting delivery (51). Thus, inhaled oxytocin and vasopressin can enter the bloodstream in humans in sufficient levels to exert clinically meaningful hormonal effects. However, in the present study, we found no evidence of entry of vasopressin into the blood following

intranasal administration of 1 μg; there was no significant increase in plasma concentration, nor any effect on blood pressure or heart rate (effects that would be expected from both central and peripheral administration of the peptides). It is likely therefore that the present method of administration (i.e. direct topical application of drops of vasopressin-containing solution to the nasal cavity of anaesthetised rats) does not favour access into the lungs in the way that deep inhalation does. It is most likely that the eventual fate of vasopressin administered in this way is to be incorporated into mucus and broken down in the gastrointestinal tract after swallowing; it is hard to see any other fate that would not allow access into the bloodstream.

Recent studies have reported that, in humans, behaviour can be affected if vasopressin or oxytocin is delivered intranasally (3,11,52). The only study that has reported measurements of either peptide in human CSF after intranasal application found that, after intranasal administration of 80 IU of vasopressin (160 μg), there was a peak concentration of approximately 40 pg/ml in the CSF (and approximately 80 pg/ml in plasma) (12). Although widely cited as evidence that intranasally-administered peptide reaches the CNS, it must be noted that the doses administered were large, even by the very generous standards of the field, yet the achieved CSF concentrations were relatively modest. Changes in CSF concentrations that cannot readily be accounted for by passage from the blood were reported, although whether the changes arise from entry into the CNS rather than from endogenous release provoked by peripheral actions of the peptide remains unresolved (53).

The blood–brain barrier protects the brain and spinal cord from a variety of pathogens and toxic substances, and presents a significant barrier for most charged and/or large molecules (54,55). This applies to most proteins and peptides, including vasopressin and oxytocin (5). In the 1970s, a large number of studies reported behavioural effects of peripherally-administered vasopressin, mainly on memory (56). However, there is convincing evidence that these can be attributed to peripheral effects of vasopressin, including vasopressor actions that, by reinforcing aversive stimulation, promoting enhanced 'memory' performance (53). A turning point was the study by Danguir (57) showing that the behavioural deficits in the (vasopressin-deficient) Brattleboro rat that could be remediated with systemic vasopressin treatment arose from disrupted sleep patterns that were normalised when normal antidiuresis was restored.

The passage of oxytocin and vasopressin from blood into brain has been carefully evaluated by Mens *et al.* (58), who estimated that, after i.v. injection in rats, just 0.002% of the injected dose reached the CNS. Some peptides that do not penetrate the blood–brain barrier have direct actions on the CNS by acting at specialised sites on the blood side of the blood–brain barrier. These sites, which comprise the so-called circumventricular organs, can be identified at: (i) the ultrastructural level by the presence of fenestrated capillaries; the (ii) light microscope level by the presence of specific biochemical markers; and (iii) functionally by the sites of penetration into the brain of dyes administered systemically. At least two of these sites, the organum vasculosum of the lamina terminalis and the subfornical organ, contain nerve fibres that are immunoreactive for vasopressin V1b receptors (59). However, these sites are not gateways into the brain for bloodborne substances; molecules that penetrate into these organs do not penetrate further into the brain.

Some drugs enter the brain readily after intranasal application, although this does not in itself imply that there is any privileged access to the brain for all molecules via this pathway. Not only do inhaled substances access the lungs swiftly, but also the nasal cavity itself has a rich vascular plexus that may permit rapid entry of topically-administered drugs. Thus, for many medications, the rates of absorption and plasma concentrations after intranasal application are similar to those achieved by i.v. administration, and this is particularly true for small, lipophilic molecules that can be delivered in high concentrations as aerosols. For larger molecules and peptides, it has been suggested that pathways involving nerves connecting the nasal passages to the brain are important, together with pathways involving the vasculature, CSF and lymphatic system. The CSF and lymphatic systems are interconnected; CSF is produced by the choroid plexuses, is maintained at high pressure, and drains into blood via the arachnoid and into lymph via some of the cranial nerve bundles, including the olfactory and trigeminal nerves (60). Thus, entry into the brain via the lymphatic system or nerve bundles route must proceed against the direction of bulk flow of the CSF. For some large peptides (e.g. leptin), it appears that specialised transport mechanisms may facilitate penetration (54,55). However, on the other hand, many mechanisms impair the bioavailability of intranasally delivered drugs, including nasal mucociliary clearance mechanisms (61–63) and drug-metabolising enzymes (64,65). Oxytocin and vasopressin, for example, are rapidly broken down by the enzyme oxytocinase, which is abundant in several brain regions, including the olfactory bulb (66).

A few studies have used autoradiography to measure the brain distribution of radiolabel after the intranasal administration of radiolabelled peptides. These studies failed to find evidence of peptide entry into the CSF, although they did report penetration of the radiolabel into brain sites approximately according to their distance from the nose, with highest concentrations of radiolabel found in the olfactory bulb (67,68). Because these studies did not use high-performance liquid chromatography to confirm the identity of the radiolabel carrier, it is not established whether the presence of the radiolabel in brain areas reflects the penetration of intact peptide, metabolites or free label. We used fluorescently-tagged vasopressin and found labelling at diverse sites throughout the brain within 20 min after intranasal application. However, we found exactly the same distribution of labelling when we administered the fluorophore alone (data not shown). This suggests that, when fluorescently tagged vasopressin is administered intranasally, cleavage by endogenous enzymes in the nasal mucous (which is exceptionally rich in antiseptic enzyme activity) (69) yields free label that is subsequently found in the brain. Again, via which route the label reaches the brain (directly or via the bloodstream) remains unclear. However, the ubiquitary distribution of the label in brain tissue at sites relatively remote from the ventricular system does not favour a primary distribution via the CSF.

This raises the question of whether the effects of intranasal peptides in humans are really mediated by activation of central vasopressin and oxytocin receptors, or by activation of their receptors in peripheral tissue, which then impacts on brain activity, as shown in animal studies (53). In human studies, when subjects receive social stimuli, it is likely that this causes reactions in the viscera (especially the heart and gut), and the heart and the gut may themselves contribute to peptide plasma levels (70). The afferent branches of the vagus nerve and other visceral afferents can transfer visceral signals, and the brain may register these as emotional signals. Evidence indicates that the autonomic nervous system is influenced by both endogenous and exogenous oxytocin (71).

Finally, we should not neglect the possibility that neuropeptides applied to the nose act on the nose, especially when applied at concentrations so high that they are likely to have promiscuous actions at many other receptor types, as well as on the vasopressin receptors that are present in olfactory epithelial cells (72). The nasal mucosa is the first line of defence against inhaled toxicants: many inhaled pollutants initiate immediate nasal responses via interaction with nasal trigeminal C-fibres involving the release of a variety of potent mediators, such as peptides, which exert a variety of effects in the nasal mucosa, including vasodilation and increased blood flow, increased vascular permeability leading to edemagenesis, and mucous hypersecretion (73).

Taken together, we report a consistent failure to find any significant effects of the intranasal administration of vasopressin or oxytocin in rats on objective parameters that are strongly influenced by centrally-administered peptide. In studies of Fos expression, we tested the effects of intranasal application of 1 μg of peptide. This is approximately equivalent to the total content of the posterior pituitary gland, and equates approximately to the total amount of vasopressin secreted in 1 month by normally hydrated rats: vasopressin and oxytocin act at high-affinity G-protein coupled receptors, and are active in the periphery at circulating concentrations in the range 1–10 pg/ml. Because injections of 2 ng i.c.v. had profound effects on Fos expression at multiple brain sites known to express oxytocin and/or vasopressin receptors, we might expect that passage into the brain of as little as 0.2% of the dose administered intranasally would be reflected in positive outcomes. We also found no effects of intranasal vasopressin on anxiety-related behaviour on the elevated plus maze, a test that has been very extensively used to study the role of vasopressin in anxiety-related behaviours. By contrast to a recent study reporting behaviour changes in an open field test after intranasal administration of oxytocin in voles (28), we failed to detect any effect of intranasal vasopressin in this paradigm. We also found no effects on social behaviour in the social recognition test, although this behaviour is known to depend on the actions of vasopressin in the olfactory bulb, which is supposedly the brain site most accessible to intranasal vasopressin. We also found no effects of intranasal vasopressin on mitral cell activity when administered intranasally, even at a 100-fold higher dose. We therefore have to conclude that vasopressin and oxytocin administered at very large doses intranasally in rats do not enter the brain in amounts that are sufficient to exert clear effects.

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