British Journal of Pharmacology (2004) 142, 181–191
www.nature.com/bjp

Bv8, the amphibian homologue of the mammalian prokineticins, modulates ingestive behaviour in rats

*,[1]Lucia Negri, [1]Roberta Lattanzi, [1]Elisa Giannini, [1]Milena De Felice, [1]Antonella Colucci & [1]Pietro Melchiorri

[1]Department of Human Physiology and Pharmacology 'V Erspamer', University of Rome 'La Sapienza', P.le A. Moro 5, Rome 00185, Italy

1 The small protein Bv8, secreted by the skin of the frog *Bombina variegata*, belongs to a novel family of secreted proteins whose mammalian orthologues have been identified and named prokineticins (PK-1 and PK-2).

2 Bv8 (from 2.5 to 60 pmol) injected into the lateral ventricles of rat brain suppressed diurnal, nocturnal, deprivation-induced and neuropeptide Y-stimulated feeding and stimulated diurnal drinking. Nocturnal drinking was increased only in fasted rats.

3 PK-2 mRNA is expressed in discrete areas of the rat brain, including the suprachiasmatic nucleus (SCN), medial preoptic area (MPA) and nucleus of the solitary tract (NTS). In the SCN neurons, PK-2 mRNA is highest during the light phase of the circadian cycle and undetectable during the dark phase.

4 The G-protein-coupled receptor prokineticin receptor 2 (PKR-2), which binds Bv8 and PK-2 with high affinity, is mainly expressed in the piriform cortex, paraventricular thalamic nucleus, parataenial nucleus (PT), SCN, hypothalamic paraventricular (PVH) and dorsomedial (DMH) nuclei, arcuate nucleus (ARC) and subfornical organ (SFO) of the rat brain.

5 Bv8 microinjected into the ARC, at doses from 0.02 to 2.0 pmol during night-time or from 0.2 to 5 pmol in 24-h-fasted rats, selectively suppressed feeding without affecting drinking. When injected into the SFO, Bv8 (from 0.2 to 2 pmol) stimulated drinking but did not affect feeding. Bv8 injections into other brain areas left rat ingestive behaviours unchanged.

6 We hypothesize that PK-2-rich projections from SCN neurons to PKR-expressing ARC neurons could transmit the circadian rhythm of feeding, whereas inputs from the PK-2-expressing NTS neurons to the PKR-2-expressing SFO neurons could transmit visceral information on the water–electrolyte balance and osmotic regulation.
British Journal of Pharmacology (2004) **142,** 181–191. doi:10.1038/sj.bjp.0705686

Keywords: Bv8; prokineticins; feeding; drinking

Abbreviations: ARC, arcuate nucleus of the hypothalamus; AT_1, angiotensin II receptor 1; DMH, dorsal medial nucleus of the hypothalamus; MPA, medial preoptic area; NTS, nucleus of the solitary tract; PK-1, prokineticin 1; PK-2, prokineticin 2; PKR-2, prokineticin receptor 2; PT, parataenial nucleus; PVH, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; RT–PCR, reverse transcriptase–polymerase chain reaction; SCN, suprachiasmatic nucleus; SFO, subfornical organ.

Introduction

The small protein Bv8, isolated from amphibian skin (Mollay *et al*., 1999), belongs to a novel family of secreted proteins whose homologues have been found in snakes (*Dendroaspis polylepsis*: VPRA or MIT-1, Joubert & Strydom, 1980; Schweitz *et al*., 1999), rodents (mouse: mBv8 or prokineticin 2 (mPK-2), Wechselberger *et al*., 1999; rat: prokineticin 1 and 2 (rPK-1, rPK-2), Masuda *et al*., 2002) and humans (prokineticin 1 (PK-1) or EG-VEGF and prokineticin 2 (PK-2), Li *et al*., 2001; LeCouter *et al*., 2001). Common structural motives in this peptide family are the amino-terminal sequence of 20 amino-acid residues and the five disulphide bonds that link the 10 cysteine residues and fold the molecules into a globular form. The distribution of mRNAs of murine Bv8-like proteins has been reported in the brain, spinal cord, gastrointestinal tract, endocrine glands and other peripheral organs of mice and rats (Wechselberger *et al*., 1999; LeCouter *et al*., 2001; Li *et al*., 2001; Melchiorri *et al*., 2001; Cheng *et al*., 2002; Masuda *et al*., 2002). In the brain, the main localizations of mPK-2 include the olfactory bulb, cerebral cortex (in some neurones of layer II), dorsal and ventral hippocampus, medial preoptic area of the hypothalamus, suprachiasmatic nucleus (SCN), some thalamic nuclei, Purkinje cells of cerebellum, sensory and motor nuclei of the brain stem and spinal cord. Using *in situ* hybridization, Cheng *et al*. (2002) found that mPK-2 mRNA is rhythmically expressed in the SCN, according to the 24-h cycle of the circadian clock. This

*Author for correspondence; E-mail: lucia.negri@uniroma1.it
Advance online publication: 5 April 2004

circadian rhythm of mPK-2 mRNA expression is severely blunted in mutant mice deficient in Clock or Cryptochrome genes.

Receptors for PK-1 and PK-2 have been identified in humans, mice (Lin *et al*., 2002) and rats (Masuda *et al*., 2002). These receptors, named prokineticin receptor 1 and 2 (PKR-1 and PKR-2), belong to the family of G-protein-coupled receptors, share approximately 85% amino-acid identity and are about 80% identical to the previously described mouse orphan receptor gpr73 (Parker *et al*., 2000). In mouse brain, PKR-2 mRNA is expressed in the lateral septal (LS) nucleus, paraventricular, parataenial and paracentral thalamic nuclei (PVT/PT/PC), paraventricular, dorsal medial and arcuate hypothalamic nuclei (PVH/DMH/ARC), suprachiasmatic nucleus (SCN), subfornical organ (SFO), lateral globus pallidus and amygdala (Cheng *et al*., 2002). In previous studies, we showed that these receptors are also expressed in rat spinal cord and dorsal root ganglia, bind Bv8 with high affinity, and, when activated by Bv8, produce $[Ca^{+2}]_i$ transients in cultured dorsal root ganglion neurones (Negri *et al*., 2002).

The neurobiology of Bv8 and its mammalian orthologues remains largely unexplored. Bv8 injections in rats produce hyperalgesia and peripheral nociceptive sensitization (Mollay *et al*., 1999; Negri *et al*., 2002), induce drinking (Giannini *et al*., 2001) and elicit antidiuretic effects (Lattanzi *et al*., 2001). In a recent study, Cheng *et al*. (2002) showed that PK-2 delivered into the rat lateral brain ventricles suppressed the high level of wheel running behaviour associated with the dark phase of the circadian cycle, and hypothesized that PK-2 controls the behavioural (locomotor) circadian rhythm from the SCN. If PK-2 transmits the SCN circadian clock, PK-2 and its amphibian homologue Bv8 could also modulate other rodent circadian behaviours, such as feeding and drinking.

In this study, we therefore investigated the effects of Bv8 on spontaneous food and water intake, and on fasting-induced and neuropeptide Y (NPY)-stimulated feeding in rats. We first studied changes in rat ingestive behaviour produced by i.c.v. injections of Bv8. To determine the neuronal pathways involved in these ingestive responses, we then mapped PK-2 mRNA and PKR-2 mRNA in the rat brain. Finally, to detect the brain nuclei involved in the feeding and drinking responses, we microinjected Bv8 into the PKR-2-expressing sites and measured the changes in food and water intake.

Methods

Animals

Male Sprague–Dawley rats (Charles River, Como, Italy), weighing 220–240 g, housed in individual plastic cages, were used for the experiments under protocols approved by the Animal Care and Use Committee of the Italian Ministry of Health according to EC directives. All animal studies were conducted in rats maintained on a 12 h light/12 h dark cycle in a temperature- ($22\pm2^{\circ}$C) and humidity- ($52\pm2\%$) controlled environment. Tap water and standard pelletted food were freely available in the home cages. Body weights were recorded each day.

Intracerebroventricular (i.c.v.) injections

Under ketamine–xylazine anaesthesia (60–10 mg kg^{-1}, i.p.), each rat was implanted surgically with a plastic guide cannula (Linca, Tel-Aviv, Israel), stereotaxically inserted through a skull hole drilled over the left lateral ventricle of the brain (AP $= -1$ mm, L $= +1.8$ mm relative to the bregma). The cannula was screwed into the skull hole and secured to bone with dental cement. After operation, rats were allowed to recover for at least 7 days before any experimental manipulation and were handled and weighed daily to habituate them to the partial restraint experienced during injections. Drugs dissolved in saline were injected into the left lateral ventricle, in a constant volume of 2 μl, using a Hamilton 5-μl syringe fitted with a 26-gauge needle that was inserted through the guide cannula to a depth of 4.2 mm below the external surface of the skull. When the experiments ended, 1 μl of methylene blue solution was injected into ventricles before the rat was killed and blue diffusion into the ventricular space was evaluated. Only rats that showed complete filling with dye of the lateral and third ventricular lumen, and diffusion of blue into the periventricular structures of the cortex, thalamus and hypothalamus were included in this study.

Intracerebral (i.c.) injections

After 1 week of adaptation to housing conditions, rats were implanted unilaterally with 26-gauge thin wall intracranial guide cannulae (Plastics One, C315G model) under ketamine–xylazine anaesthesia. Rats were placed in a Kopf stereotaxic instrument with the incisor bar positioned 3.5 mm above the interaural line and a 0.5-mm hole was drilled in the rat skull to insert guide cannulae 0.3 mm above the targets at the following brain sites: SFO, using coordinates -0.92 mm posterior to bregma (AP), 0.0 lateral to the midline (ML) and 4.7 mm ventral to the skull surface (DV); PVH (anterior parvicellular part), using coordinates AP -1.6 mm, ML 0.4 mm and DV 7.5 mm; PVH (lateral magnocellular part), using coordinates AP -1.8 mm, ML 0.6 mm and DV 7.5 mm; DMH, using coordinates AP -3.3 mm, ML 0.5 mm and DV 8.3 mm; ARC, using coordinates, AP -2.8 mm, ML 0.3 mm and DV 9.1 mm; lateral globus pallidus, using coordinates, AP –2.2 mm, ML 4.0 mm and DV 6.7 mm; basomedial amygdala, using coordinates, AP –2.8 mm, ML 4.1 mm and DV 8.2 mm; and basolateral amygdala, using coordinates AP –2.8 mm, ML 5.0 mm and DV 8.2 mm (Paxinos & Watson, 1998). At the completion of surgery, cannulae were fitted with 28-gauge inner stylets (Plastics One, C315DC model) to maintain patency. For microinjections into the target nuclei, a 33-G stainless-steel tube (inner cannula, Plastics One, C315I model) was connected to a 10-μl SGE syringe *via* 30-cm PE50 tubing and the assembly was filled with the solution to be injected. The syringe was mounted on a microprocessor-controlled microsyringe pump (World Precision Instruments, UMP2 model) and the pump was programmed to deliver 0.5 μl of fluid at a rate of 0.25 μl min^{-1}. Rats were gently restrained while the inner cannula was inserted into the guide cannula and the injection was administered.

The extent of Bv8 diffusion within brain extracellular space was evaluated by two approaches: (i) by assessing spread of [^{125}I]Bv8 injected into the medial hypothalamic area by autoradiography; (ii) by measuring drinking and feeding

responses to 2 pmol Bv8 dose injected 0.6–1.5 mm distant from the target nuclei or to 0.2 pmol dose injected into the third ventricle. In nine rats bearing intracranial guide cannulas (AP –2.30 mm, ML 1.2 mm, DV 8.8 mm), [^{125}I]Bv8 (2.0 pmol, 0.1 μCi , 0.5 μl at rate of 0.25 μl min^{-1}) was injected into the brain *via* a syringe pump as described previously. Rats were killed 15, 30 and 60 min after injection and the brain was quickly removed, immediately frozen in chilled isopentane (−40°C) and kept at –80°C until sliced with a cryostat. Slicing started 3 mm anterior to and ended 3 mm posterior to the injection site. Adjacent sections of 50-μm thickness were mounted on glass slides and the slides were apposed to X-ray film. Autoradiograms were quantitatively analysed using Versadoc 3000 imaging system (Bio-Rad, Milan, Italy). With the aid of a computer image analysis software (MCID Elite, Imaging Research, Canada), the extent of [^{125}I]Bv8 diffusion was estimated in the three dimensions of space by plotting the optical density (OD) as a function of the distance from the injection site (Figure 7b).

To evaluate the spatial localization of Bv8 effects after injection into periventricular nuclei, we compared the drinking and feeding responses to 2 pmol Bv8 injected 0.6–1.5 mm away from the SFO and ARC (Figure 7a: open circles) or to 0.2 pmol Bv8 injected into the third ventricle, with the responses evoked by the same doses injected into the SFO and ARC. For third ventricular injections, an intracranial guide cannula was implanted over the third ventricle (AP −1.8 mm, ML 0.0 mm, DV 3.1 mm) and Bv8 solution (0.5 μl) was injected at a rate of 0.25 μl min^{-1} to a depth of 4.4 mm, using the microprocessor-controlled microsyringe pump UMP2.

Intracerebral and third ventricular injection sites were verified by histological examination of the rat brains at the end of the study. In brief, the animals were injected with 0.5 μl of sonicated India ink solution and decapitated. The brains were rapidly removed and snap-frozen in liquid nitrogen using isopentane as a cryopreservative. A freezing cryostat was used to take 20-μm sections and the sections were stained with cresyl violet to allow anatomical localization. An expert observer blind to treatment conditions and behavioural data verified the histological localization. For i.c. injections, the deepest site containing India ink as identified by histology was taken as the site of injection. In the experiments with third ventricular injections, the complete filling of the third ventricular lumen with India ink was assured. The histological analysis showed correct cannula placements in 63 of 93 rats in the SFO, in 112 of 152 rats in the ARC, in 21 of 29 rats in the SCN, in 22 of 31 rats in the PVH, in 21 of 26 rats in the DMH, in seven of 10 rats in the amygdala and in eight of 10 rats in the globus pallidus. Rats that received 2 pmol Bv8 injections into sites 0.6–1.5 mm away from the SFO and ARC were used in experiments to evaluate functionally the spatial localization of Bv8 effects.

Food and water intake

After i.c.v. or i.c. injections, rats were returned to their cages, where preweighed amounts of food pellets and water were made available. At predefined time points, remaining food and spillage was measured to the nearest 0.01 g, and the weight of eaten food was corrected for spillage. To evaluate water intake, each animal was allowed to drink water from a glass bottle calibrated to 0.1 ml and fitted with stainless-steel metal drinking spouts. Latency to drink was determined as the time from the presentation of water until the rat licked sufficiently at the spout to cause a bubble to rise.

Gene expression analysis of PK-2 and PKR-2 mRNA by in situ *hybridization and reverse transcriptase-polymerase chain reaction (RT–PCR)*

To localize PK-2 and PKR-2 mRNA in the rat brain, we used an *in situ* hybridization technique with specific riboprobes. In brief, we generated antisense and sense riboprobes containing the coding region of rat PK-2 and PKR-2 (GenBank accession numbers AY089984 and AY089975; Melchiorri *et al*., 2001), labelled them with 35S and processed *in situ* hybridization as described by Seidman (1999). Radioactivity was detected by coating slides (20-μm sections) with Hypercoat LM-1 emulsion (Amersham Biosciences, Milan, Italy). Slides were counterstained with thionin solution. Anatomical localization and regional nomenclature were defined according to Paxinos & Watson (1998). Specific hybridization signals in the brain nuclei were quantitatively analysed using a video-based computer image analysis system (MCID Elite, Imaging Research, Canada). A calibration curve of OD *versus* radioactivity (d.p.m. mg^{-1} tissue wet weight) was constructed using ^{14}C standards.

Several hypothalamic and extrahypothalamic areas of the rat brain expressing PK-2 and PKR-2 mRNA were microdissected according to the Palkovits method (Palkovits & Browstein, 1983). Frozen sections, 300-μm thick, were examined under a stereomicroscope with 20-fold magnification and 0.2–0.5 mm tissue discs were removed from the chosen brain regions with a hollow punching needle. For each selected brain nucleus, a punch small enough to fit into the nucleus was used to dissect the target nucleus, while negative control samples were taken from closely neighbouring brain areas. After punching, the 300-μm brain slices were mounted on a specimen holder and 20-μm sections were cut in a cryostat, fixed and stained to validate the dissected sites. Tissue samples were immediately placed in 100 μl of cold RNA extraction solution (buffer RLT, Qiagen, Milan, Italy) and extracted RNA was purified using RNeasy columns (Qiagen, Milano, Italy), and checked for accidental degradation on agarose gel. Purified RNA (2 μg) were used for cDNA synthesis with reverse transcriptase (Promega, Milan, Italy) and the reaction product was diluted to 100 μl with deionized water. An aliquot of 2.5 μl of cDNA solution was used for PCR amplifications (Robocycler 40, Stratagene, Florence, Italy). To compensate for variations in RNA quantitation and random tube-to-tube variations, a multiplex PCR reaction was performed in which the level of product from the gene of interest was normalized against the product from 18S ribosomal RNA, assumed as an internal control. Primers for 18S ribosomal RNA amplification were purchased from Ambion (Milan, Italy). Based on the Primer-Competimer TM Technology (Ambion), PCR amplification conditions were the same as for targets. Specific sense and antisense primers were synthesized (Biogen, Rome, Italy) to PCR amplify the rat PK-2 and PKR-2 cDNA, according to the following sequences: PK-2, 5′-TCATCACCGGGGCTTGCG-3′ and 5′-TAACTTTCCGAGTCAGGG-3′; PKR-2, 5′-TCTCCTTGCTCCCCTTAAAC-3′ and 5′-AGATGGGATGGCGATGAG-3′. Ribosomal 18S RNA was used as

internal standard. PCR products were separated by electrophoresis on agarose gel, stained with ethidium bromide and the resulting fluorescent bands were revealed and measured with the Versadoc 3000 imaging system (Bio-Rad, Milan, Italy), excised from the gel, eluted (GFXTM PCR kit, Amersham Pharmacia, Italy) and sequenced (Primm, Milan, Italy) to confirm the expected products.

For each sample, the density of bands on agarose gel was divided by the density of the 18S RNA band and expressed as the relative density (RD).

Experimental protocols

Bv8 activity on rat ingestive behaviour was studied according to the following protocols.

Rat circadian ingestive behaviour A week after cannula implantation, the basal 12 h light/dark consummatory behaviour of each rat was recorded daily for another week before the experiments started. Only rats showing stable circadian ingestive behaviour were used. On 2 consecutive days before experiments, all rats received, in random order, two daily sham or vehicle injections, one injection 2 h after light on and the other injection at light off, to habituate them to the drug administration procedure. Sham injections were administered exactly as vehicle injections, including insertion of syringe needle or inner cannula into the cranial guide cannula, but no fluid was delivered. I.c.v. doses of Bv8 ranged from 0.25 to 60 pmol per rat. Bv8 doses injected into hypothalamic nuclei were 10 pmol into the PVH, DMH and SNC; from 0.02 to 2 pmol into the SFO; and from 0.02 to 5 pmol into the ARC. Each rat received Bv8 injection i.c.v. or i.c. only once.

To study ingestive behaviour during the light phase, groups of seven rats each were injected 2 h after light on and cumulative food and water intake was measured for 4 and 3 h, respectively. In drinking experiments, rats received, in random order, vehicle or one dose of Bv8. Feeding behaviour was studied on 2 consecutive days: the same rats received saline injection the first day and Bv8 injection the next day, thus serving as their own controls. Chow was not available during drinking experiments and water was not available during feeding experiments. In separate rat groups, we compared the effect of i.c.v. injection of Bv8 with that of angiotensin II (ANG) on drinking behaviour and tested the effects of the angiotensin II receptor 1 (AT_1) antagonists, losartan (5 μg, i.c.v.) and EXP73174 (0.5 μg, i.c.v.), and of the V_1 (H5350, 20 ng, i.c.v.) and the V_2 (H7707, 5 ng, i.c.v.) vasopressin receptor antagonists, on water intake produced by i.c.v. Bv8 (25 pmol).

To study ingestive behaviour during the night-time, the same treatment protocols described for diurnal drinking and feeding experiments were used. Rats were injected at the time of light off, and immediately returned to their cages, where preweighed amounts of food pellets and water were made available. Cumulative food and water intake was measured during the dark phase for 12 h. To avoid meal-associated drinking, separate rat groups were deprived of food for 20 h and i.c.v. injected with Bv8 (from 0.5 to 25 pmol) or vehicle at light off. Their nocturnal drinking behaviour was measured for 12 h and compared with that of ad libitum-feeding rats.

Food intake in 24-h fasted rats All experiments began 2 h after light on. Rats, deprived of food for 24 h, were injected, in random order, with vehicle or one dose of Bv8 and returned to their home cages along with preweighed amounts of food. Food intake was measured for 3 h. Bv8 was injected i.c.v. (from 0.5 to 60 pmol) or into ARC (from 0.02 to 5 pmol).

NPY-induced feeding All experiments began 2 h after light on. At 1 h before the experiment, animals were deprived of food and water. Feeding was induced by injections of 1 nmol NPY into the left lateral ventricle. Bv8 or vehicle were injected i.c.v. 30 min before NPY. This interval was selected because of the latency to the Bv8 effect. Immediately after NPY injections, rats were returned to their cages along with preweighed amounts of food. Food intake was measured for 3 h.

Drugs Bv8 was extracted from the skin secretion of electrically stimulated *Bombina variegata*, purified to a single peak by RP-HPLC (Mollay *et al*., 1999) and lyophilized. Lyophilized Bv8 was analysed by the electrospray ionization mass spectrometry (Mariner System 5220 mass spectrometer, Applied Biosystem, U.S.A.) to confirm the calculated molecular mass of the protein (8020.18 DA) and the presence of five disulphide bonds. The lyophilized protein was 98% pure, containing about 2% of water. [^{125}I]Bv8 (specific activity 2200 Ci/mmol) was prepared by labelling Bv8 with [^{125}I] Bolton–Hunter reagent as described previously (Negri *et al*., 2002). NPY was purchased from Calbiochem Biochemicals and ANG from Tocris Cookson, Bristol, U.K.; H5350 and H7707 were purchased from Bachem, Switzerland; losartan and EXP73174 were kind gifts from Professor Maurizio Massi, Camerino, Italy.

Statistical evaluation The data are presented as mean $\pm$ s.e. mean values. In the experiments on rat circadian ingestive behaviour, the time course of food consumption by each rat group after vehicle (first day) and Bv8 (second day) injection was analysed by repeated-measures two-way ANOVA (time and treatment). *Post hoc* Bonferroni test was used to compare means of Bv8- and vehicle-injected rat groups, for each dose and at each time point. Changes in food intake by each rat after Bv8 were then calculated for each dose and time point, by subtracting the food eaten after Bv8 injection from food eaten after vehicle injection and data were analysed by two-way ANOVA and means were compared by *post hoc* Bonferroni test. Water intake as well as food intake in 24-h-fasted and NPY-injected rats were analysed by two-way ANOVA (time and treatment) followed by Bonferroni test. Data on vasopressin and angiotensin antagonists and circadian expression levels of PK-2 were analysed by *t*-test. Statistical significance was set at $P<0.05$.

Results

Effects of i.c.v.-injected Bv8 on circadian ingestive behaviour

Day-time feeding No significant difference was found in the amount of food that vehicle-injected rats ate over the 4-h observation period during the light phase and during the same

period when they received sham i.c.v. injections (2.6±0.25 *versus* 2.1±0.11 g). I.c.v. injections of Bv8 decreased food intake (Figure 1a): 5 pmol was the lowest Bv8 dose that significantly decreased diurnal food intake during the first 2 h, whereas the 60-pmol dose decreased cumulative food consumption over 4 h.

Night-time feeding Bv8 injected i.c.v. when the dark phase of the circadian cycle began resulted in an intense inhibition of food intake. The lowest dose (0.25 pmol) significantly reduced the nocturnal food intake over the first 2 h but not in the subsequent 10 h (Figure 1b). Higher doses significantly reduced feeding throughout the dark phase, so that Bv8-injected rats ate smaller cumulative amounts of nocturnal food than they had eaten on the previous night, when they received i.c.v. injections of the vehicle. During the dark phase, the difference in the cumulative amount of food eaten by Bv8- and vehicle-treated rats changed in a dose-dependent manner: after the 0.25 pmol dose, it remained constant for the first 2 h and then rapidly declined to nonsignificant values within the third hour; after the 2.5 pmol dose it reached a maximum at the third hour and then decreased slowly during the second half of the night; and after the 5.0 and 25.0 pmol doses, it increased until the ninth hour of the dark phase (Figure 1c).

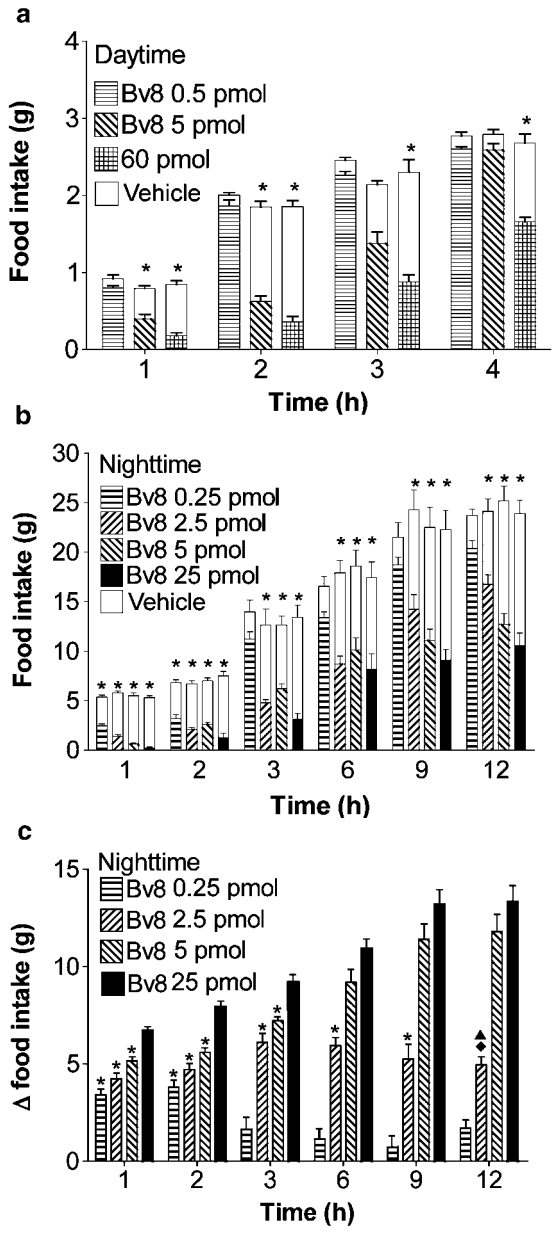

Figure 1 Effects of i.c.v. injections of Bv8 on food intake during the light phase (a) and the dark phase (b and c) in freely fed rats. Within each group, the same rats received vehicle injection the first day, and Bv8 injection the next day, thus serving as their own controls. Results are shown as cumulative food intake (g, mean± s.e.m.) of seven rats per group in (a) and (b) and as a difference (Δg, mean±s.e.m.) in the cumulative amount of food eaten by Bv8-treated rats. (c) Results in (a) and (b) were analysed by repeated-measures two-way ANOVA and in (c) by two-way ANOVA. *Post hoc* Bonferroni test: (a) and (b) $^*P<0.05$ *versus* vehicle-injected rats; (c) $^*P<0.05$ *versus* 25 pmol Bv8-injected rats.

In two separate rat groups, we injected i.c.v. 750 pmol of human recombinant PK-1 ($n=4$) and 50 pmol of human recombinant PK-2 ($n=4$) and measured the nocturnal food intake. Both prokineticins inhibited nocturnal feeding with a potency and time course similar to the 25 pmol dose of Bv8 (data not shown).

Day-time drinking Bv8-injected rats drank significantly more than vehicle-injected rats (after 5 pmol Bv8, 2.3±1 ml; after 60 pmol Bv8, 7.6±1.3 ml). A Bv8 dose of 360 pmol did not further increase the volume drunk (8.1±1.6 ml). After Bv8 injection, latency to drink was 24.2±4.4 min, and all doses yielded their maximum dipsogenic response within 2 h (Figure 2a).

Rats drank a similar maximum volume of water after 60 pmol Bv8 and after 50 pmol ANG. The latency to drinking was far longer after Bv8 than after ANG (24.6±5 min and about 1 min). I.c.v. pretreatment with the angiotensin AT_1 receptor antagonists losartan (5 μg, −5 min) and EXP73174 (0.5 μg, −5 min) left the dipsogenic effect of Bv8 (60 pmol) unchanged, whereas it completely inhibited the increase in water intake produced by i.c.v. administration of 50 pmol ANG (Table 1). The AV_1 vasopressin receptor antagonist H5350 (20 ng, i.c.v., −10 min) and the AV_2 vasopressin receptor antagonist H7707 (5 ng, i.c.v., −10 min), left the Bv8-induced dipsogenic response unchanged (Table 1).

Night-time drinking *Ad libitum*-feeding rats drank a large volume of water (27±1 ml) during the dark phase of the circadian cycle. In these rats, no significant difference was found in the absolute amount of drunk water between vehicle-injected and 5 or 25 pmol Bv8-treated animals. Only the highest Bv8 dose tested (60 pmol), which maximally stimulated drinking during the light phase, significantly increased drinking during the night-time (Figure 2b). If chow was not available during the night, vehicle-injected rats delayed drinking to the third hour and drank a reduced amount of water (18±0.4 ml, $P<0.05$ *versus ad libitum*-feeding rats). Fasting rats injected with Bv8 showed an early and large increase in drinking: a Bv8 dose of 5 pmol significantly increased water intake during the first 3 h of the dark phase and 25 pmol dose stimulated drinking throughout the dark phase (Figure 2c).

Locomotion, body temperature and weight After the i.c.v. dose of 360 pmol Bv8, the rats appeared calm and sleepy. If stimulated or put in an open field, however, they walked

normally and their rota-rod performance resembled that of controls (data not shown). In two distinct groups of four rats each, rectal temperature and body weight were measured before (0) and 15, 30, 60, 120 and 240 min after i.c.v. injection of vehicle ($n=4$) and 60 pmol Bv8 ($n=4$). A slight decrease in body temperature was observed only 60 min after Bv8 injection (0 min, vehicle 36.9±0.2°C, Bv8 37.1±0.2; 60 min, vehicle 37.0±0.2°C, Bv8 36.6±0.3). No change in body temperature was recorded 120 and 240 min after Bv8 injection. Body weight did not change significantly over the period examined.

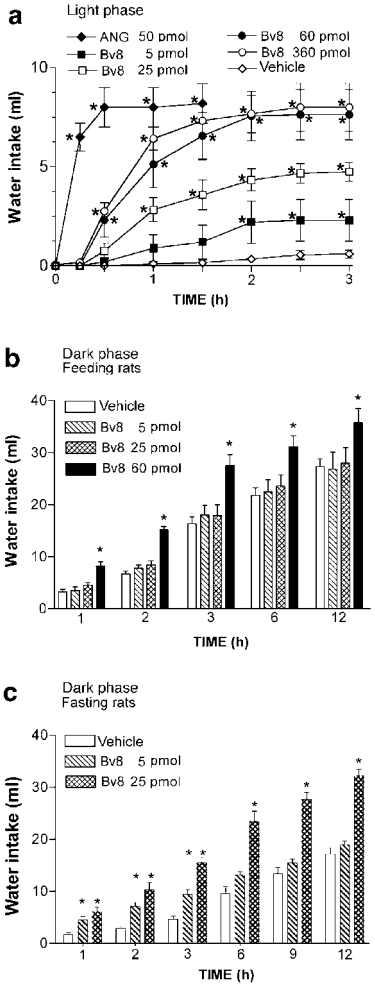

Figure 2 Cumulative water intake in rats after i.c.v. administration of graded doses of Bv8. Results are shown as cumulative water intake (ml, mean±s.e.m.) of seven rats per group. Two-way ANOVA with *post hoc* Bonferroni test: $*P<0.05$ *versus* vehicle-injected rats. (a) Water intake during the light phase. For comparison, the time course of mean dipsogenic response to i.c.v. ANG of five rats is shown. (b) Water intake of *ad libitum*-feeding rats during the dark phase; and (c) water intake of fasting rats during the dark phase.

Table 1 A 90-min water intake (ml±s.e.m.) induced by i.c.v. injection of Bv8 and ANG, in rats pretreated with saline, AT_1 receptor antagonists (losartan and EXP73174) and antagonists of vasopressin receptor V_1 (H5350) and V_2 (H7707)

Pretreatment	*Saline*	*ANG, 50* pmol	*Bv8, 60* pmol
Saline	0.71±0.2	8.2±0.9	7.7±0.91
Losartan, 5.0 μg, i.c.v.	0.75±0.2	0.5±0.1	7.9±0.97
EXP73174, 0.5 μg, i.c.v.	0.42±0.1	0.2±0.1	8.5±1.05
H5350, 20.0 ng, i.c.v.	0.57±0.1	—	9.2±1.10
H7707, 5.0 ng, i.c.v.	0.48±0.2	—	8.5±0.70

Effects of i.c.v.-injected Bv8 on fasting-induced food intake

The diurnal food intake induced by preceding 24-h fasting was dose-dependently reduced by i.c.v. injections of Bv8. I.c.v. doses of 2.5, 12 and 60 pmol of Bv8 inhibited feeding by 38, 73 and 92% during the first hour, and by 24, 37 and 70% over the 3-h period (Figure 3a).

Effects of i.c.v.-injected Bv8 on NPY-induced food intake

NPY (1 nmol) injected into the brain lateral ventricles stimulated feeding significantly over the 4-h period (7.0±0.7 g). Pretreatment (−30 min) with Bv8 reduced both first hour and cumulative 3-h food intake (15 pmol, by 53 and 40%; 30 pmol, by 75 and 50%; 50 pmol, by 81 and 76%, *versus* saline) (Figure 3b).

Expression of PK-2 and PKR-2 mRNA in the rat brain

PK-2 and PKR-2 mRNAs were expressed in several hypothalamic and extrahypothalamic areas of the rat brain. Analysis of PK-2 mRNA distribution by *in situ* hybridization on 20-μm sections of rat brains taken during the light-phase showed that PK-2 mRNA was mainly expressed in the hypothalamic nuclei

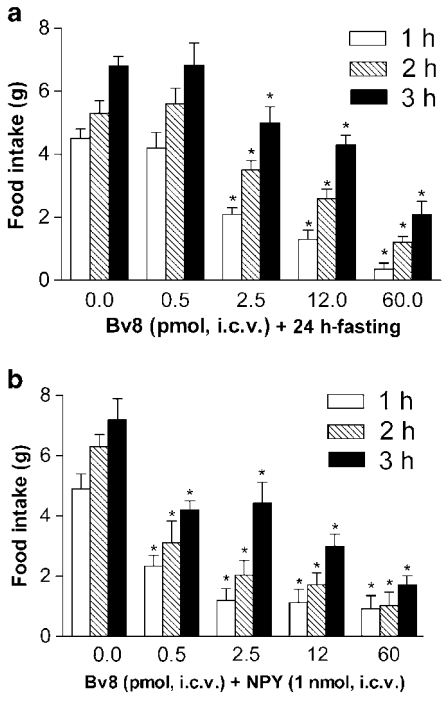

Figure 3 Effects of i.c.v. injections of graded doses of Bv8 on food intake in 24h-fasting rats (a) or rats stimulated by i.c.v. NPY (b), during day-time. Results are shown as cumulative food intake (g, mean±s.e.m., $n=7$). One-way ANOVA with *post hoc* Bonferroni test: (a) $*P<0.05$ *versus* vehicle-injected rats (0.0); (b) $*P<0.05$ *versus* NPY-injected rats.

SCN and medial preoptic area (MPA) and the hind brain nucleus of solitary tract (NTS), among a few other discrete brain areas. Brain sections taken during the dark phase showed that PK-2 mRNA was absent in SCN, but apparently increased in MPA and in NTS (Figure 4a and b). An intense expression of PKR-2 mRNA was found by *in situ* hybridization analysis in the piriform cortex (PIR), SFO, PVH, DMH, ARC, SCN, PVT and PT (Figure 4c and d). PKR-2 mRNA is also expressed in many nuclei of the extended amygdala, hippocampus, lateral habenular nucleus and lateral globus pallidus (data not shown).

RT–PCR amplification of mRNA extracted from microdissected brain areas detected transcripts of PK-2 in samples from SCN, MPA and NTS. In samples taken during the night-time, PK-2 mRNA was undetectable in SCN and apparently increased in MPA and NTS (Figure 5a and c). Transcripts of the prokineticin receptor PKR-2 were detected in samples from the PVH, PVT, SCN, SFO, DMH and ARC (Figure 5b and c), but none in samples from closely neighbouring areas of the hypothalamus.

Effects of intrahypothalamic injections of Bv8

Feeding Bv8 injected into the ARC significantly reduced nocturnal food intake and feeding stimulated by 24-h fasting (Figure 6a and b). The lowest Bv8 intranuclear dose that significantly reduced nocturnal feeding was 0.02 pmol. Diurnal feeding stimulated by 24-h fasting was significantly inhibited by a dose of 0.2 pmol Bv8. Microinjections of 10 pmol Bv8 into the SCN, PVH, DMH, SFO, amygdala and globus pallidus left food intake unchanged (data not shown).

Drinking Bv8 injected into rat SFO increased diurnal water drinking (Figure 6c). The lowest active Bv8 intranuclear dose was 0.2 pmol and maximum drinking (8.2 ± 0.9 ml) was obtained with 2.0 pmol. Latency to drink was significantly shorter after injection into SFO than after i.c.v. injection (4.6 ± 1.3 *versus* 24.2 ± 4.4; *t*-test, $P < 0.05$). Injections of 10 pmol Bv8 into the SCN, PVH, DMH, ARC, amygdala and globus pallidus left diurnal water intake unchanged (data not shown).

Spatial extent of intrahypothalamic-injected Bv8 effects No difference was found in nocturnal feeding and diurnal drinking of rats injected with 2 pmol Bv8 into brain sites from 0.6 to 1.5 mm away from the ARC and SFO (Figure 7a) and vehicle-injected rats. Injection of 0.2 pmol Bv8 into rat third ventricle left diurnal drinking unchanged, and slightly but not significantly decreased nocturnal feeding during the first hour of the night.

Autoradiographic assessment of Bv8 spread Plotting the [^{125}I]Bv8 radioactivity as a function of the distance from the injection site (Figure 7b) demonstrated that drug radioactivity

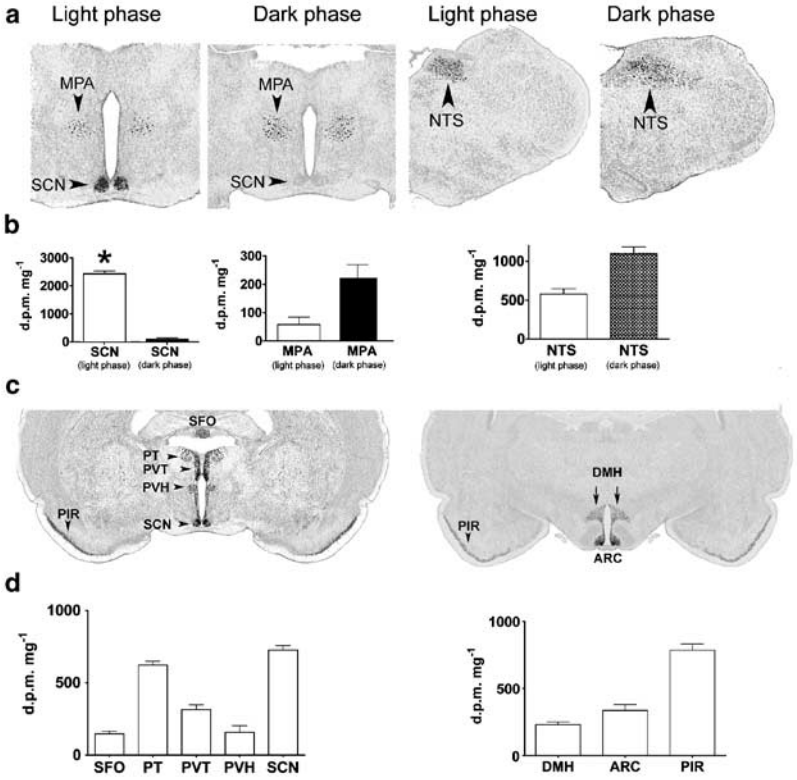

Figure 4 Localization of PK-2 and PKR-2 mRNAs in the rat brain by *in situ* hybridization analysis. (a) Localization of PK-2 mRNA in the MPA, SCN and NTS in brain sections taken during day-time and night-time. (b) Quantitative analysis of PK-2 mRNA expression by *in situ* hybridization (d.p.m. mg^{-1}, mean $\pm$ s.e.m., $n = 4$). PK-2 mRNA expression in the SCN during the light phase was compared with that during the night phase (*$P < 0.05$, *t*-test): (c) Localization of PKR-2 mRNA in the PIR, SFO, PT and PVT nuclei, hypothalamic paraventricular (PVH), SCN, DMH and ARC nuclei: (d) Quantitative analysis of PKR-2 mRNA expression by *in situ* hybridization (d.p.m. mg^{-1}, mean $\pm$ s.e.m., $n = 4$).

decreased exponentially from the centre of the injection site to the periphery, achieving a maximum radius of 800–1000 μm 15 min after injection. The peak radioactivity levels decreased slowly after injection, being approximately 70% of the 15 min value after 30 min and 50% after 60 min. These results show that Bv8 has a slow diffusion rate within brain extracellular

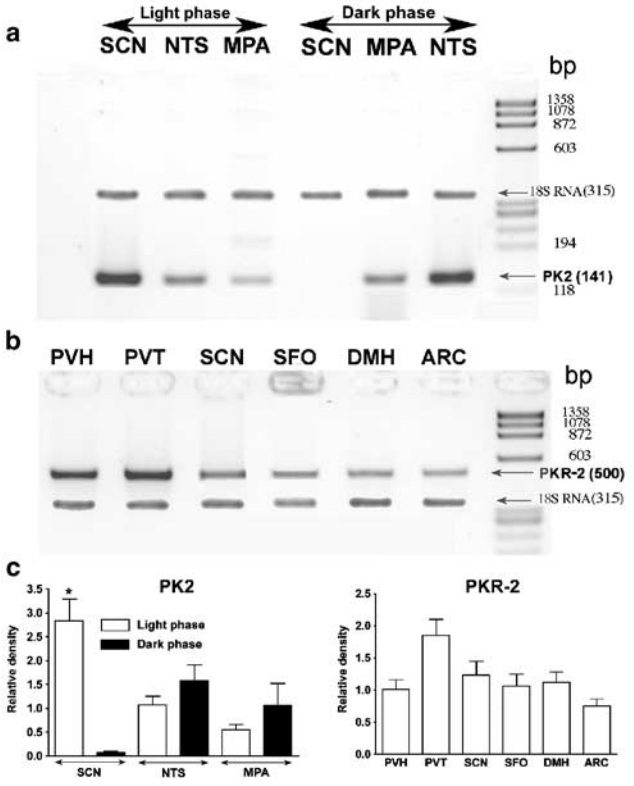

Figure 5 Agarose-gel electrophoresis of RT–PCR products of PK-2 (a), PKR-2 (b) mRNAs from the following microdissected rat brain areas: SCN, MPA, NTS, SFO, PVT, PVH, SCN, DMH and ARC nuclei. PK-2 transcripts are from the same brain areas microdissected during the light phase and dark phase of the circadian cycle. The right lane indicates the molecular markers for DNA fragments, and the transcripts of PK-2 mRNA (141 bp), PKR-2 mRNA (500 bp) and ribosomal 18S RNA. OD of transcript bands relative to 18S RNA band density are plotted in (c) for all the microdissected brain areas (RD, mean ± s.e.m., $n = 4$).

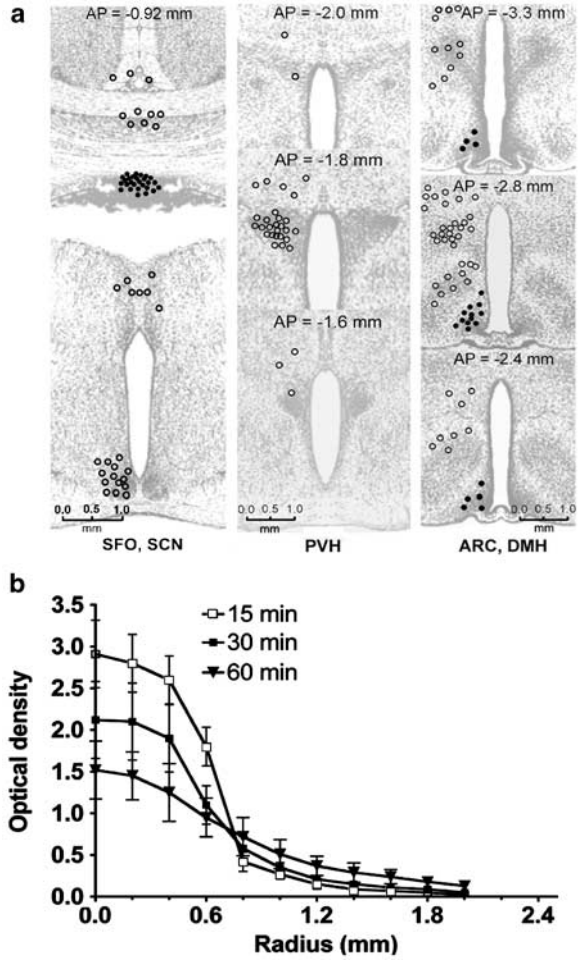

Figure 7 Histological reconstruction of intrahypothalamic injection sites for the 2 pmol Bv8 dose (a) and autoradiographic diffusion profile (b) of 2 pmol [^{125}I]Bv8 injected into the rat hypothalamus. In (a), filled circles show sites where 2 pmol Bv8 injections produced significant dipsogenic (SFO) or anorexigenic (ARC) responses. Open circles show injection sites where Bv8 injections left rat food and water intake unchanged. In (b), OD (mean ± s.e.m. $n = 3$ rats) of the autoradiographic spots, produced by 2 pmol [^{125}I]Bv8 injected into medial hypothalamic area, is plotted as a function of distance from the site of injection.

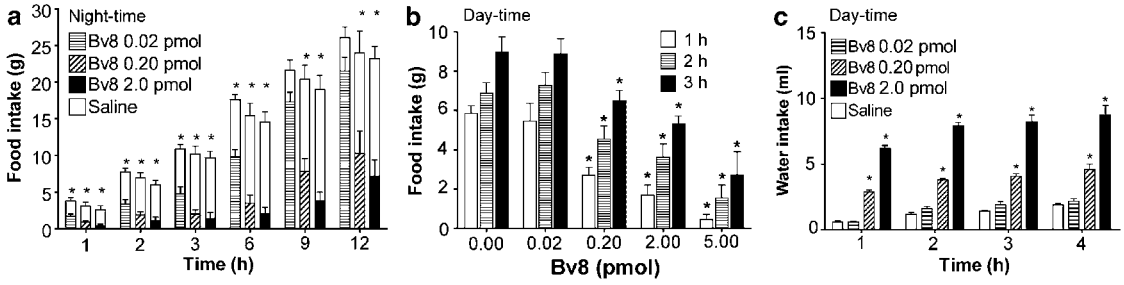

Figure 6 Effects of Bv8 microinjections into the ARC (a and b) and SFO (c) on rat feeding (a and b) and drinking (c). Results are shown as cumulative food or water intake (g or ml, mean ± s.e.m.) of seven rats per group. (a) Night-time food intake of rats that received saline injection the first day and Bv8 injection the next day, thus serving as their own controls (repeated-measures two-way ANOVA with *post hoc* Bonferroni test: $^*P < 0.05$ *versus* vehicle-injected rats). (b) Day-time feeding of 24-h-fasting rats that received, in random order, either saline or Bv8. Two-way ANOVA with *post hoc* Bonferroni test: $^*P < 0.05$ *versus* vehicle-injected rats. (c) Day-time drinking of rats injected with either saline or graded doses of Bv8. Two-way ANOVA with *post hoc* Bonferroni test: $^*P < 0.05$ *versus* vehicle-injected rats.

space and a maximum spherical spread of 800–1000 μm radius for an injected volume of 0.5 μl at a constant rate of 0.25 μl min^{-1}.

Discussion

In this study, Bv8 injected into the lateral ventricles of rat brain effectively suppressed day-time, night-time, deprivation-induced and NPY-stimulated feeding. In preliminary experiments, recombinant human PK-2, the mammalian orthologue of Bv8, also displayed potent dipsogenic and anorexigenic activity. These results overall confirm that Bv8 reproduces the effects of mammalian prokineticins not only on angiogenesis (LeCouter *et al*., 2001), intestinal motility (Li *et al*., 2001) and nociception (Negri *et al*., 2002) but also on drinking and feeding.

In this study, examination by *in situ* hybridization analysis of rat brain cryosections and RT–PCR amplification of mRNA, extracted from microdissected samples of rat brain areas, showed that the SCN, MPA and NTS are the main nuclei expressing PK-2 and the SCN, SFO, PVH, DMH and ARCH are the hypothalamic areas richest in PKR-2 mRNA. These results confirm previous *in situ* hybridization experiments on the localization of PK-2 and PKR-2 mRNA in mouse brain (Cheng *et al*., 2002). As we had already demonstrated that Bv8 binds the prokineticin receptors with high affinity (Negri *et al*., 2002), the expression of PKR-2 in a hypothalamic area involved in the control of ingestive behaviour supports the hypothesis that Bv8 modulates rat feeding and drinking by activating prokineticin receptors in these hypothalamic neurones.

Numerous studies using either discrete lesions in the hypothalamus or surgical transection of neural pathways (Kalra *et al*., 1999) demonstrated that the ARC contains neural mechanisms affecting ingestive behaviour. The ARC is critical for the maintenance of body energy balance (Elmquist *et al*., 1999, Schwarz *et al*., 2000). Destruction of the ARC leads to profound metabolic disturbances (Olney, 1969), and genetic defects in ARC function have been linked to obesity in humans (Barsh *et al*., 2000). The PVH and ARC are also considered the main sites for the regulation of food intake by NPY (Kalra *et al*., 1991). In this study, Bv8 injected into rat ARC, at doses lower than the i.c.v. threshold doses, strongly inhibited night-time- and deprivation-induced feeding. Conversely, microinjections of Bv8 into other hypothalamic and extrahypothalamic brain sites, including those expressing Bv8-prokineticin receptors and placed 0.6–1.5 mm away from the ARC, had no effect on food intake. Finally, our autoradiographic studies demonstrated that hypothalamic-injected [^{125}I]Bv8 diffused slowly from the injection site within an injection radius of 800–1000 μm and the radius of the diffusion sphere remained practically unchanged over the 60-min period examined. These results ensure that the observed anorexigenic response to intra-ARC injection of Bv8 resulted from the activation of prokineticin receptors in the ARC and not in other adjacent nuclei.

Among central inputs to the ARC are signals coming from the SCN, MPA and NTS whose neurones express PK-2, the mammalian orthologue of amphibian Bv8. In rodents, feeding behaviour undergoes the SCN-controlled circadian rhythm (Saeb-Parsy *et al*., 2000). Rodents eat during the dark phase of the circadian cycle and the expression of PK-2, in mouse SCN neurons, oscillates in a circadian manner and responds to light entrainment with a huge increase to signal the beginning of the 'circadian light period' (Cheng *et al*., 2002). In the present experiments, we confirmed that PK-2 mRNA is rhythmically expressed also in the rat SCN neurons and provided evidence that Bv8 injected into the ARC was significantly more potent in suppressing feeding during the dark phase of the circadian cycle, when PK-2 expression in SCN neurons was undetectable, than during the light period. During the circadian light period, PK-2, highly expressed in SCN could therefore act as an output molecule that transmits the circadian light signal of the SCN clock to the ARC to suppress feeding. Conversely, during the night-time the presumed low level of PK-2 in the ARC could sensitize ARC neurons to the anorexigenic action of exogenous Bv8.

The mechanism by which Bv8/prokineticins inhibit feeding in the ARC is still unknown. NPY is among the prime modulators of feeding that act on ARC neurons. In this study, Bv8 antagonized the orexigenic response produced by exogenous NPY. Whether it inhibited spontaneous feeding by blocking endogenous NPY activity remains unclear. PKR-2 was cloned 2 years before the discovery of prokineticins as an orphan receptor closely related to the Y-receptor family (Parker *et al*., 2000). Neither PKR-2 bound NPY, PYY or PP nor did prokineticins or Bv8 bind any member of the Y-receptor family (data not shown). Hence the Bv8-induced inhibition of NPY-induced food intake that we described in this study could arise from a mechanism other than antagonism at Y-receptors. The anorexigenic effects of Bv8 may result from other direct or indirect actions on the ARC neurones or arcuate-hypothalamic projections that modulate feeding. For example, Bv8 can release other anorexigenic peptides from the ARC neurones, such as alpha-MSH, CART peptide, corticotropin-releasing hormone, urocortin III, cholecystokinin, glucagon-like peptides and neurotensin, or inhibit the release of peptides that stimulate food intake, such as NPY, agouti-related peptide, orexins, melanin concentrating hormone and galanin. Bv8 can act on intrahypothalamic neuronal circuits that exist between these peptidergic neurones including the arcuate-paraventricular and arcuate-dorsolateral hypothalamic projections.

Electrophysiological, immunohistochemical and microdialysis studies are underway to identify the neuronal pathways on which Bv8/prokineticins act, to characterize hypothalamic neurons expressing PKs and PKRs and to ascertain the release of hypothalamic neuropeptides and neurotransmitters.

The SFO is considered one of the circumventricular organs of rat brain connected with neural mechanisms affecting thirst. Together with the vascular organ of the lamina terminalis (OVLT), the SFO has also been recognized as a site of action for ANG to stimulate drinking in rats (McKinley *et al*., 1992). In rats, another dipsogenic peptide that stimulates water intake in response to hyperosmotic signals is vasopressin (Stricker *et al*., 2002). In a previous study, we showed that Bv8 stimulates the release of vasopressin into the rat blood thus producing antidiuretic effects (Lattanzi *et al*., 2001). As PKRs are expressed in many nuclei of the extended amygdala, which show moderate angiotensin immunoreactivity and in SCN and PVH rich in vasopressin, Bv8-induced drinking could result from angio-

tensin and vasopressin release from these brain sites. The results we obtained with antagonists of angiotensin and vasopressin receptors nevertheless indicate that the two neuropeptides do not mediate Bv8 dipsogenic activity. Accordingly, microinjections of Bv8 into rat SFO strongly stimulated water intake, whereas injections into other hypothalamic nuclei and in adjacent sites did not. These results and the autoradiographic analysis of the spread of intrahypothalamic Bv8 indicate that Bv8 dipsogenic activity could result from a direct activation of PKRs localized on SFO neurones. In the rat, the SFO receives afferent neuronal inputs from the SCN, MPA and NTS (Lind *et al*., 1982; Zardetto-Smith & Watson, 1987; Larsen *et al*., 1991), whose neurones express high levels of PK-2 mRNA.

In preliminary experiments reported in the present study, using human recombinant PKs, we demonstrated that also these mammalian orthologues of Bv8 display potent dipsogenic effects in rats. The NTS receives afferent sensory input from the GI tract in processing information related to water–electrolyte balance and osmotic regulation during feeding (Johnson & Thunhorst, 1997; Schreihofer *et al*, 1999). Thus, at least in the rat, NTS neurones could control meal-associated water intake by releasing PK-2 from their neuronal terminals projecting to the SFO (Starbuck *et al*., 2001) thereby activating PKRs. In the present experiments, during night-time, Bv8 stimulated drinking more potently in fasting rats than in *ad libitum*-feeding rats: the presumed low level of PK-2 in the SFO, due to the lack of meal-associated drinking signals, could sensitize fasting rats to the dipsogenic action of exogenous Bv8.

Once selective PKR antagonists and antibodies against PKs and PKRs are available, it will be interesting to examine the involvement of the mammalian orthologues of the amphibian Bv8 in the regulation of ingestive behaviour as well as in the SCN-controlled circadian ingestive rhythm and NTS-mediated osmotic control. Adding these small secreted proteins to the long list of neuropeptides that modulate ingestive behaviour would make the brain mechanisms regulating appetite, satiety and thirst more complex to understand. Yet, it might also help to unravel the complex mechanism regulating these behaviours.

We thank Dr Giuseppina Mignogna for Bv8 purification and Dr Napoleone Ferrara (Genentech, U.S.A.) for the kind gift of EG-VEGF (PK-1). This work was supported by grants from the Italian Ministry of University and Scientific Research (National Research Projects) and from the University of Rome, 'La Sapienza', Italy.

References

BARSH, G.S., FAROOQI, I.S. & O'RAHILLY, S.O. (2000). Genetics of body weight regulation. *Nature,* **404,** 644–651.

CHENG, M.J., BULLOCK, C.M., LI, C., LEE, A.G., BERMAK, J.C., BELLUZZI, J., WEAVER, D.R., LESLIE, F.M. & ZHOU, Q.Y. (2002). Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature,* **417,** 405–410.

ELMQUIST, J.K., ELIAS, C.F. & SAPER, C.B. (1999). From lesions to leptin: hypothalamic control of food intake and body weight. *Neuron,* **22,** 221–232.

GIANNINI, E., LATTANZI, R. & NEGRI, L. (2001). The novel peptide Bv8 regulates fluid intake in rats. *Pharmacological Res.,* **43** (A), 29.

JOHNSON, A.K. & THUNHORST, R.L. (1997). The neuroendocrinology of thirst and salt appetite: visceral sensory signals and mechanisms of central integration. *Front. Neuroendocrinol.,* **18,** 292–353.

JOUBERT, F.J. & STRYDOM, D.J. (1980). Snake venom. The amino acid sequence of protein A from *Dendroaspis polylepsis* (black mamba) venom. *Hoppe Seylers Z. Physiol. Chem.,* **361,** 1787–1794.

KALRA, S.P., DUBE, M.G., PU, S., XU, B., HORWATH, T.L. & KALRA, P.S. (1999). Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr. Rev.,* **20,** 68–100.

KALRA, S.P., DUBE, M.G., SAHU, A., PHELPS, C.O. & KALRA, P.S. (1991). Neuropeptide Y secretion increases in the paraventricular-nucleus in association with increased appetite for food. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 10931–10935.

LARSEN, P.J., MOLLER, M. & MIKKELSEN, J.D. (1991). Efferent projections from the periventricular and medial parvicellular subnuclei of the hypothalamic paraventricular nucleus to circumventricular organs of the rat.: a *Phaseolus vulgaris* leusoagglutinin (Pha-L) tracing study. *J. Comp. Neurol.,* **306,** 462–479.

LATTANZI, R., GIANNINI, E. & NEGRI, L. (2001). Bv8, a small protein from frog skin induces antidiuresis in rats. *Pharmacol. Res.,* **43** (A), 30.

LECOUTER, J., KOWALSKI, J., FOSTER, J., HASS, P., ZHANG, Z., DILLARD-TELM, L., FRANTZ, G., RANGELL, L., DEGUZMAN, L., KELLER, G.A., PEALE, F., GURNEY, A., HILLAN, K.J. & FERRARA, N. (2001). Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature,* **412,** 876–884.

LI, M., BULLOCK, C.M., KNAUER, D.J., EHLERT, F.J. & ZHOU, Q.Y. (2001). Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Mol. Pharmacol.,* **59,** 692–698.

LIN, D.C.H., BULLOCK, C.M., EHLERT, F.J., CHEN, J.L., THIAN, H. & ZHOU, Q.Y. (2002). Identification and molecular characterization of two closely related G-protein coupled receptors activated by prokineticins/EG-VEGF. *J. Biol. Chem.,* **277,** 19276–19280.

LIND, R.W., VAN HOESEN, G.W. & JOHNSON, A.K. (1982). An HRP study of the connections of the subfornical organ of the rat. *J. Comp. Neurol.,* **210,** 265–277.

MASUDA, Y., TAKATSU, Y., TERAO, Y., KUMANO, S., ISHIBASHI, Y., SUENAGA, M., ABE, M., FUKUSUMI, S., WATANABE, T., SHINTANI, Y., YAMADA, T., HINUMA, S., INATOMI, N., OHTAKI, T., ONDA, H. & FUJINO, M. (2002). Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochem. Biophys. Res. Commun.,* **293,** 396–402.

MCKINLEY, M.J., BICKNELL, R.J., HARDS, D., MCALLEN, R.M., VIVAS, L., WEISINGER, R.S. & OLDFIELD, B.J. (1992). Efferent pathways of the lamina terminalis subserving osmoregulation. *Prog. Brain Res.,* **91,** 395–402.

MELCHIORRI, D., BRUNO, V., BESONG, G., NGOMBA, R.T., CUOMO, L., DEBLASI, A., COPANI, A., MOSCHELLA, C., STORTO, M., NICOLETTI, F., LEPPERDINGER, G. & PASSARELLI, F. (2001). The mammalian homologue of the novel peptide Bv8 is expressed in the central nervous system and supports neuronal survival by activating the MAP kinase/PI-3-kinase pathways. *Eur. J. Neurosci.,* **13,** 1694–1702.

MOLLAY, C., WECHSELBERGER, C., MIGNOGNA, G., NEGRI, L., MELCHIORRI, P., BARRA, D. & KREIL, G. (1999). Bv8, a small protein from frog skin and its homolog from snake venom induce hyperalgesia in rats. *Eur. J. Pharmacol.,* **374,** 189–196.

NEGRI, L., LATTANZI, R., GIANNINI, E., METERE, A., COLUCCI, M.A., BARRA, D., KREIL, G. & MELCHIORRI, P. (2002). Nociceptive sensitization by the secretory protein Bv8. *Br. J. Pharmacol.,* **137,** 1147–1154.

OLNEY, J.W. (1969). Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. *Science,* **164,** 719–721.

PARKER, R., LIU, M., EYRE, H.J., COPELAND, N.G., GILBERT, D.J., CRAWFORD, J., SURHERLAND, G.R., JENKINS, N.A. & HERZOG, H. (2000). Y-receptor-like genes GPR72 and GPR73: molecular cloning, genomic organization and assignment to human chromosome 11q21.1 and 2p14 and mouse chromosome 9 and 6. *Biochem. Biophys. Acta,* **1491,** 369–375.

PALKOVITS, M. & BROWSTEIN, M.J. (1983). *Brain Microdissection Technique*. Great Britain: John Wiley & Sons, pp. 1–36.

PAXINOS, G. & WATSON, C. (1998). *The Rat Brain in Sterotaxic Coordinates*. Academic Press: U.S.A.

SAEB-PARSY, K., LOMBARDELLI, S., KHAN, F.Z., MCDOWALL, K., AU-YONG, I.T. & DYBALL, R.E. (2000). Neural connections of hypothalamic neuroendocrine nuclei in the rat. *J Neuroendocrinol.,* **12,** 635–648.

SCHREIHOFER, A.M., ANDERSON, B.K., SCHILTZ, J.C., XU, L., SVED, A.F. & STRICKER, E.M. (1999). Thirst and salt appetite elicited by hypovolemia in rats with chronic lesions of the nucleus of the solitary tract. *Am. J. Physiol.,* **276,** R251–R258.

SCHWARZ, M.W., WOODS, S.C., PORTE JR, D., SEELEY, R.J. & BASKIN, D.G. (2000). Central nervous system control of food intake. *Nature,* **404,** 661–671.

SCHWEITZ, H., BIDARD, JN. & LAZDUNSKI, M. (1999). Purification and pharmacological characterization of peptide toxins from the black mamba (*Dendroaspis polylepsis*) venom. *Toxicon,* **28,** 847–856.

SEIDMAN, J.G. (1999). Current Protocols in Molecular Biology. Vol. 3, 14.3.1–14.3.10. U.S.A.: John Willey & Sons, Inc.

STARBUCK, E.M. & FITTS, D.A. (2001). Influence of the subfornical organ on meal- associated drinking in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **280,** R669–R677.

STRICKER, E.M., HUANG, W. & SVED, A.F. (2002). Early osmoregulatory signals in the control of water intake and neurohypophyseal hormone secretion. *Physiol. Behav.,* **76,** 415–421.

WECHSELBERGER, C., PUGLISI, R., ENGEL, E., LEPPERDINGER, G., BOITANI, C. & KREIL, G. (1999). The mammalian homologues of frog Bv8 are mainly expressed in spermatocytes. *FEBS Lett.,* **462,** 177–181.

ZARDETTO-SMITH, G. & WATSON, C. (1987). A direct neural projection from the nucleus of the solitary tract to the subfornical organ in the rat. *Neurosci. Lett.,* **80,** 163–166.

(Received October 9, 2003
Revised December 20, 2003
Accepted January 7, 2004)