

Abnormal fluid homeostasis in apelin receptor knockout mice

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Abstract

The apelinergic system, comprised of apelin and its G protein-coupled receptor (APJ; APLNR as given in MGI Database), is expressed within key regions of the central nervous system associated with arginine vasopressin (AVP) synthesis and release as well as in structures involved in the control of drinking behaviour, including the magnocellular neurones of the hypothalamus, circumventricular organs, and the pituitary gland. This localisation is indicative of a possible functional role in fluid homeostasis. We investigated a role for APJ in the regulation of fluid balance using mice deficient for the receptor. Male APJ wild-type and knockout (APJ^{-/-}) mice were housed in metabolic cages to allow determination of water intake and urine volume and osmolality. When provided with free access to water, APJ^{-/-} mice drank significantly less than wild-types, while their urine volume and osmolality did not differ. Water deprivation for 24 h

significantly reduced urine volume and increased osmolality in wild-type but not in APJ^{-/-} mice. Baseline plasma AVP concentration increased comparably in both wild-type and APJ^{-/-} mice following dehydration; however, APJ^{-/-} mice were unable to concentrate their urine to the same extent as wild-type mice in response to the V2 agonist desmopressin. Analysis of *c-fos* (*Fos* as given in MGI Database) mRNA expression in response to dehydration showed attenuation of expression within the subfornical organ, accentuated expression in the paraventricular nucleus, but no differences in expression in the supraoptic nucleus nor median pre-optic nucleus in APJ^{-/-} mice compared with wild-type. These findings demonstrate a physiological role for APJ in mechanisms of water intake and fluid retention and suggest an anti-diuretic effect of apelin *in vivo*.

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Introduction

Apelin, a novel 36 amino acid peptide originally isolated from extracts of bovine stomach (Tatemoto *et al.* 1998), mediates its actions through the G protein-coupled (GPCR) apelin receptor (APJ; APLNR as given in the MGI Database). In addition to apelin-36, other possible isoforms of the peptide, including the pyroglutamyl form of apelin-13 (pGlu-apelin-13) and apelin-17, have been discovered in rat hypothalamic extracts and plasma (De Mota *et al.* 2004). Synthetic peptides pGlu-apelin-13 and apelin-17 bind to APJ and exhibit greater biological activity than the parent peptide on the human (Habata *et al.* 1999, Zou *et al.* 2000, Medhurst *et al.* 2003) or rat APJ (De Mota *et al.* 2000). In recent years cDNA sequences for human, rat, and mouse APJ have been determined (O'Dowd *et al.* 1993, Devic *et al.* 1999, O'Carroll *et al.* 2000), with closest identity (31%) found to the angiotensin II (AngII) receptor, type AT1a.

The apelinergic system has a widespread but selective expression in the central nervous system (CNS) implicating it in the regulation of a number of physiological processes (Lee *et al.* 2000, O'Carroll *et al.* 2000, Reaux *et al.* 2002).

Significantly, *APJ* mRNA expression and apelin immunoreactivity are present in magnocellular neurones of the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON), sites that control the hypothalamic–neurohypophysial system (HNS), the pituitary gland and within the circumventricular organs (CVOs, structures involved in the control of drinking behaviour; De Mota *et al.* 2000, Reaux *et al.* 2001, 2002, Brailoiu *et al.* 2002, O'Carroll & Lolait 2003).

The key CNS site integrating neuroendocrine adjustments to water homeostasis is the hypothalamic PVN. Arginine vasopressin (AVP), a major neurohypophysial hormone within the HNS, is synthesised in magnocellular neurones of the hypothalamic PVN and SON (Burbach *et al.* 2001) that project through the median eminence to the neurohypophysis and form the hypothalamo–neurohypophysial tract (Antunes-Rodrigues *et al.* 2004). The HNS is the primary source of AVP and the related peptide oxytocin (OT) that are secreted into the systemic circulation from neurones terminating in the posterior pituitary. These magnocellular neurones are themselves innervated by osmosensitive neurones originating in the CVOs, including the organum vasculosum of the lamina terminalis (OVLT), subfornical

organ (SFO), median pre-optic nucleus (MnPO), and median pre-optic area. Elevated plasma osmolality and/or decreased plasma volume, as seen during periods of water deprivation, stimulate activity within the CVOs–HNS resulting in the secretion of AVP into the circulation (Antunes-Rodrigues *et al.* 2004, McKinley *et al.* 2004). In the kidney plasma AVP acting at the AVP V2 receptor increases renal collecting cell permeability via increased translocation, synthesis, and expression of the water channel aquaporin-2, so promoting water retention (Knepper & Inoue 1997).

Previous studies have demonstrated expression of APJ to be differentially regulated in rats in response to fluid deprivation and increased dietary salt within brain areas such as the PVN and SON, indicating a role for the apelinergic system in fluid homeostasis (O'Carroll & Lolait 2003). In addition, in the neurohypophysial axis, the physiological effects of apelin appear to be mediated by AVP but inconsistent effects have been reported. There is evidence that apelin acts to counteract AVP actions through regulation of AVP neurone activity and AVP secretion (Reaux *et al.* 2001, De Mota *et al.* 2004, Reaux-Le Goazigo *et al.* 2004, Azizi *et al.* 2008). Additionally, a study on water deprivation showed a significant increase in both the number and labelling intensity of magnocellular apelin-immunoreactive cells in the rat SON and PVN that were significantly reduced after chronic exposure to the AVP V1 receptor antagonist MeT-AVP (1- β -mercapto- β , β -cyclopentamethylene-propionyl)-2-(*O*-methyl)Tyr,Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂; Reaux-Le Goazigo *et al.* 2004). Repeated i.c.v. injections of AVP resulted in an increase in SON and PVN apelin-immunoreactivity, an effect that could be attenuated by i.c.v. pretreatment with the AVP V1 antagonist, indicating that this effect, like the dehydration-induced effects, is mediated through AVP V1 receptors. Exogenously administered apelin also reduced the amount of circulating AVP and stimulated diuresis (De Mota *et al.* 2004). Contrary to these studies are reports that *in vitro* apelin-13 significantly stimulated AVP release from rat hypothalamic explants (Taheri *et al.* 2002), while apelin-13 administration onto SON neurones via microdialysis increased the firing rates of AVP cells (Tobin *et al.* 2008). In this latter study, apelin inhibited both basal and stimulated somatodendritic release of AVP. Apelin and APJ have therefore been linked to the regulation of body fluid homeostasis, in particular to a role regulating the anti-diuretic action of AVP (Lee *et al.* 2000, Reaux *et al.* 2001, 2002, Taheri *et al.* 2002, De Mota *et al.* 2004, Reaux-Le Goazigo *et al.* 2004, Azizi *et al.* 2008), but many contradictions exist that remain to be resolved.

Similarly, a role for the apelinergic system in thirst and drinking behaviour has been proposed, although currently reports also differ with regard to its contribution. In water replete (WR) animals an immediate, short lived (30 min) but significant increase in water intake following i.p. or i.c.v. apelin injection (Lee *et al.* 2000, Taheri *et al.* 2002) has been reported, whereas other studies found apelin to significantly reduce water intake up to 12 h post i.c.v.

injection (Clarke *et al.* 2009) or to have no effect (Reaux *et al.* 2001, Mitra *et al.* 2006). Additionally, studies in water deprived (WD) animals have shown regulatory actions of apelin on fluid homeostasis with i.c.v. apelin reducing (Reaux *et al.* 2001) or having no effect (Mitra *et al.* 2006) on water intake. However, in mice lacking the apelin gene the dehydration-induced drinking response was comparable with that of wild-type mice suggesting that apelin does not have an apparent role in water intake (Kuba *et al.* 2007). A peripheral increase in apelin (i.p.) appeared to activate neurones in the CVOs and hypothalamus consistent with the involvement of the apelinergic system in fluid homeostasis (Takayama *et al.* 2007).

To date, the majority of studies have used exogenous administration of either apelin or AVP to elucidate the role of the apelinergic system in body fluid homeostasis. In this study, we have used APJ knockout (KO) mice to investigate the involvement of APJ in body fluid regulation during normal physiological conditions and water deprivation.

Materials and Methods

Animals

Adult male littermates (a mix of the C57BL/6J and 129X1/SvJ strains) of crosses using mice heterozygous for the APJ mutation (Deltagen, San Carlos, CA, USA) were bred and maintained on site. Prior to the experimental period, animals were housed under controlled light conditions (12 h light:12 h darkness cycle, lights on at 0700 h) and temperature (21 \pm 2 °C) with free access to standard lab chow and water. All procedures were conducted in accordance with the Animal Scientific Procedures Act (1986), the United Kingdom and the appropriate University of Bristol Ethical Review Process.

Offspring were genotyped at weaning using PCR analysis of DNA isolated from tail clips. Genomic templates were used for PCR analysis with primers directed to the APJ coding sequence (Primers 1, 2, 6, and 7) and to the neomycin resistance gene (contained in the disrupted targeting vector construct; Primers 3, 4, and 5) in a buffer mix: 10 \times Hot Start buffer (Continental Lab Products, Northampton, UK; 1 μ l), dNTP mix (Perkin Elmer; 1.6 μ l), primer mix (for Primers 3 and 4: 0.2 μ l of 10 μ M of each primer; for all other Primers: 1.0 μ l of 4 μ M of each primer), CLP Hot start polymerase (0.2 μ l), and dH₂O (to a final volume of 10 μ l). Oligonucleotide primers used were as follows: Primer 1 (5'-TGATGGTTACAACACTACTATGGGGCT-3') and Primer 2 (5'-GTCAAACCTCCCGGTAGGTATAAGTG-3') were used to amplify the wild-type allele, Primer 3 (5'-AGAGGCTATTTCGGCTATGACTG-3') and Primer 4 (5'-TTCGTCCAGATCATCCTGATC-3') were used to amplify the recombinant allele and Primer 5 (5'-GGG TGGGATTAGATAAATGCCTGCTCT-3'), which was unique to the neomycin cassette, was used in a 'multiplex'

reaction with Primers 6 (5'-ATGGGGCTGACAAC-CAGTCTGAATG-3'), and 7 (5'-CACAGTGGCAAAGT-CACCACAAAGG-3') to detect both wild-type and targeted alleles. Negative controls contained no template DNA. PCR conditions were 96 °C for 15 min, followed by 35 cycles of 96 °C for 10 s, 60 °C for 30 s, 68 °C for 90 s, with a final extension of 68 °C for 7 min.

Measurement of fluid intake, urine output, and osmolality

Mice (8–14 weeks-old wild-type $n=16$; APJ KO (APJ^{-/-}) $n=16$) were individually housed in metabolic cages (Techniplast, Varese, Italy) to examine the effect of APJ deletion on fluid intake, urine output, and osmolality. Animals were weighed and allowed to acclimatise to the cage for 48 h with free access to crushed lab chow and water. All subsequent measurements and procedures were conducted between 0900 and 1030 h. Mice were given a measured amount (50 ml) of water, and spontaneously voided urine was collected under mineral oil (200 µl; Sigma) for 24 h. Basal water intake, urine output, and osmolality (freezing point depression; Roebing Osmometer, Berlin, Germany) were measured. The animals were then divided into two groups, one of which was deprived of drinking water for 24 h (WD; $n=8$), while the second group continued to have free access to water (WR; $n=8$) after which water intake, urine output, and osmolality were again measured. Mice were killed by decapitation and trunk blood collected for measurement of plasma AVP, while brains were rapidly dissected out, frozen on dry ice, and stored at -80 °C.

Effect of AVP V2-receptor agonist desmopressin

To examine the renal responses of APJ^{-/-} mice to AVP, the effect of endogenous AVP was mimicked by exogenous administration of an AVP-V2 receptor agonist, desmopressin (dDAVP; Sigma). Mice ($n=5-6$) were housed in metabolic cages as described above and, following the appropriate period of acclimatisation, baseline urine samples were collected by bladder massage. Mice were then injected subcutaneously with 1 µg/kg dDAVP or vehicle (0.9% saline) and water bottles were removed. Urine samples were collected from the cage 4 h after injection and by bladder massage 4 h after injection. Urine samples were centrifuged briefly to remove debris and osmolality measured by freezing point depression.

Measurement of plasma AVP

Trunk blood was collected in chilled tubes containing EDTA (1 mg/ml blood; Sigma) and aprotinin (500 KIU/ml blood; Sigma). Samples were kept on ice and centrifuged at 1690 g for 20 min at 4 °C. Plasma was stored at -80 °C until assay. Plasma AVP concentration was determined by specific RIA following extraction with heat-activated LiChroprep Si60 (Merck; Landgraf *et al.* 1995). The level of sensitivity was

0.1 pg/sample; cross-reactivity of the polyclonal anti-AVP antibody with structurally related peptides including lysine vasopressin and OT was <0.7%. Intra- and inter-assay coefficients of variation were in 6–9 and 9–12% ranges respectively; all plasma samples to be compared were assayed in the same run.

In situ hybridisation histochemistry

Sections (12 µm) of tissue were cut, thaw-mounted onto polysine-coated slides (VWR, Lutterworth, UK), and stored at -80 °C until hybridisation. Specific riboprobes were used to look at neurone activity within the CVOs/HNS and to confirm genotype. All ³⁵S riboprobes were generated by PCR using 129SV genomic DNA as template. For the mouse, *c-fos* (*Fos* as given in MGI Database primers (upstream 5'-ACAGATCTGCCTGCAAGATCC-3'; downstream 5'-GTGAGCTCAGGGAGTCGGAGGA-3') corresponding to 2164–2679 bp of a mouse *c-fos* (*Fos* as given in MGI Database) gene (Genbank Accession number J00370) were used to generate a 516 bp product. The primers contain the recognition sequences for the restriction endonucleases BglII and SacI. For the mouse AVP, ³⁵S riboprobe primers (upstream 5'-TGCTGCAGCGACGAGAGCTGCGT-3'; downstream 5'-GCAAGCTTATTTTCCATGCTGT-3') corresponding to bp 2966–3392 of a mouse AVP-neurophysin II gene (Genbank Accession number M88354) were used to generate a 224 bp product. The primers contain the recognition sequences for the restriction endonucleases PstI and HindIII. For the mouse APJ, ³⁵S riboprobe primers (upstream 5'-GCCCCGAATTCACCTTCATTCAGC-CATGGAAGAT-3'; downstream 5'-GTCAGGATCCCG-GTAGGTATAAGTGGCCCACAGT-3') corresponding to bp 256–549 of a mouse apelin receptor cDNA (Genbank Accession number NM 011784) were used to generate a 293 bp product. The primers contain the recognition sequences for the restriction endonucleases EcoRI and BamHI. Primer restriction endonuclease sites allowed subcloning into pGEM4Z (Promega), and sense and antisense probes were generated using T7 and SP6 polymerases (*c-fos* antisense: linearised with HindIII and generated with SP6 polymerase; sense: linearised with EcoRI and generated with T7 polymerase; AVP and APJ antisense: linearised with EcoRI and generated with T7 polymerase; sense: linearised with HindIII and generated with SP6 polymerase) with ³⁵S-UTP (Perkin Elmer) and the MAXIscript *in vitro* transcription kit (Ambion, Austin, TX, USA). The integrity of each probe was verified by DNA sequencing.

Hybridisation was carried out as previously described (<http://intramural.nimh.nih.gov/lcmr/snge/Protocols/ISHH/ISHH.html>). Slides were exposed to Amersham Hyperfilm MP film for 8 weeks for APJ (reflecting the low abundance of APJ mRNA), 2 days for AVP, and 5 weeks for *c-fos* at room temperature with appropriate ¹⁴C-labeled standards (Amersham). No specific hybridisation was detected with sense probes.

Histology

In order to compare peripheral tissue morphology of wild-type and $APJ^{-/-}$ mice, a hematoxylin and eosin (H&E) stain was carried out on 4 μm thick sections of paraffin-embedded, Bouin's (Sigma) fixed organs, including the pituitary gland, adrenal gland, kidney, lung, heart, ovary and testis. Tissue morphology was assessed using light microscopy.

Statistical analysis

Water intake and urine output were calculated as 'ml per 24 h per 20 g body weight'. All data are expressed as mean \pm S.E.M. Any differences between WR wild-type and WR $APJ^{-/-}$ mice were determined by Student's (unpaired two-tailed) *t*-test. Effects of genotype and treatment were determined by two-way ANOVA with repeated measures and subsequent *post hoc* tests. Plasma AVP RIA data are expressed as pg/ml, given as mean \pm S.E.M., and compared using two-way ANOVA. For *in situ* hybridisation histochemistry (ISHH) data, analysis of the autoradiographic images of probe bound and visualised on film was carried out using NIH Image software (Rasband, version 1.22; <http://rsb.info.nih.gov/nih-image/>) run on an Apple Macintosh IICi using 2–4 sections from each animal. ISHH was carried out on tissue sections cut from regions of interest (SFO, MnPO, SON, PVN). These regions were delineated by comparison of $APJ^{-/-}$ images to wild-type control images cut at the same tissue level. For each image, the region of interest was selected and its mean optical density determined. Using the standard curve, a representative value for that area was obtained; data were expressed as a percentage of the mean for the control group (wild-type/WR) and this was used for statistical analysis. All values to be statistically compared were processed in the same assay to avoid inter-assay variability. ISHH data are expressed as a percentage of the mean for the control group (wild-type/WR), and compared using two-way ANOVA and *post hoc* tests. Statistical comparisons were made using GraphPad Prism software. $P < 0.05$ was considered statistically significant.

Results

Characteristics of $APJ^{-/-}$ mice

Mice lacking the APJ gene were bred from APJ heterozygous matings. Absence of the gene in homozygous mutant mice was determined by PCR (Fig. 1A). Analysis of progeny genotypes at weaning showed 6% to be homologous for the disrupted allele, 28% to be wild-type and 66% to be heterozygous for the APJ mutation, indicating a loss of homozygous mutants. However, homozygous survivors were viable and fertile and, besides a significantly lower body weight than wild-type littermates ($APJ^{-/-}$ 26.71 \pm 0.68 g, wild-type 28.44 \pm 0.50 g, $P < 0.05$), showed no obviously visible phenotype. Histological analysis of H&E-stained sections from the

pituitary gland, adrenal gland, kidney and lung (Fig. 1B) and heart, ovary and testis (data not shown), did not reveal any apparent morphological differences in $APJ^{-/-}$ mice compared with wild-type littermates. Representative ISHH autoradiographic images showing *APJ* mRNA expression in sections of heart and PVN from wild-type and $APJ^{-/-}$ mice are shown in Fig. 1C. No *APJ* mRNA expression was observed in $APJ^{-/-}$ mice. Expression of *APJ* mRNA by ISHH was not detected in other peripheral $APJ^{-/-}$ tissues including the lung and kidney (data not shown).

Role of APJ in fluid homeostasis in mice with free access to drinking water

Under normal conditions (WR) drinking behaviour, measured as intake in 24 h, was significantly less in $APJ^{-/-}$ mice compared with wild-type (wild-type 5.3 \pm 0.5 ml/24 h per 20 g; $APJ^{-/-}$ 3.9 \pm 0.4 ml/24 h per 20 g; $P < 0.05$). Spontaneously voided urine volume (wild-type 1.00 \pm 0.12 ml/24 h per 20 g; $APJ^{-/-}$ 1.02 \pm 0.14 ml/24 h per 20 g) and urine osmolality (wild-type 3008 \pm 165 mOsmol/kgH₂O; $APJ^{-/-}$ 3169 \pm 252 mOsmol/kgH₂O) were comparable between genotypes (Fig. 2).

Effect of water deprivation on fluid homeostasis in wild-type and $APJ^{-/-}$ mice

Water deprivation for 24 h significantly decreased urine volume in wild-type mice (0.954 \pm 0.20 vs 0.375 \pm 0.09 ml/24 h per 20 g; $P < 0.01$) but not in $APJ^{-/-}$ mice (0.891 \pm 0.18 vs 0.668 \pm 0.15 ml/24 h per 20 g; $P > 0.05$; Fig. 3). Similarly, urine osmolality was significantly increased in wild-type (3284 \pm 246 vs 5229 \pm 389 mOsmol/kgH₂O; $P < 0.05$) but not in $APJ^{-/-}$ mice (3649 \pm 351 vs 4610 \pm 674 mOsmol/kgH₂O; $P > 0.05$).

Plasma AVP

In WR mice, plasma AVP levels tended to be lower in $APJ^{-/-}$ mice (23.3 \pm 6.3 pg/ml) than in wild-type (39.5 \pm 7.8 pg/ml), although this did not achieve statistical significance ($P = 0.138$). Following 24 h WD, there were comparable increases in AVP concentration in both genotypes (wild-type 57.7 \pm 3.5 pg/ml versus $APJ^{-/-}$ 52.9 \pm 2.9 pg/ml; Fig. 4).

Effect of dDAVP on urine osmolality

Urine osmolalities prior to dDAVP administration were comparable between wild-type and $APJ^{-/-}$ mice. Administration of dDAVP significantly increased urine osmolality both in wild-type (3278 \pm 258–4635 \pm 244 mOsmol/kgH₂O; $P < 0.01$) and $APJ^{-/-}$ mice (2575 \pm 322–3590 \pm 151 mOsmol/kgH₂O; $P < 0.05$), however the dDAVP-induced increase in $APJ^{-/-}$ mice was significantly attenuated compared with wild-type ($APJ^{-/-}$ 3590 \pm 151 versus wild-type 4635 \pm 244 mOsmol/kgH₂O; $P < 0.05$).

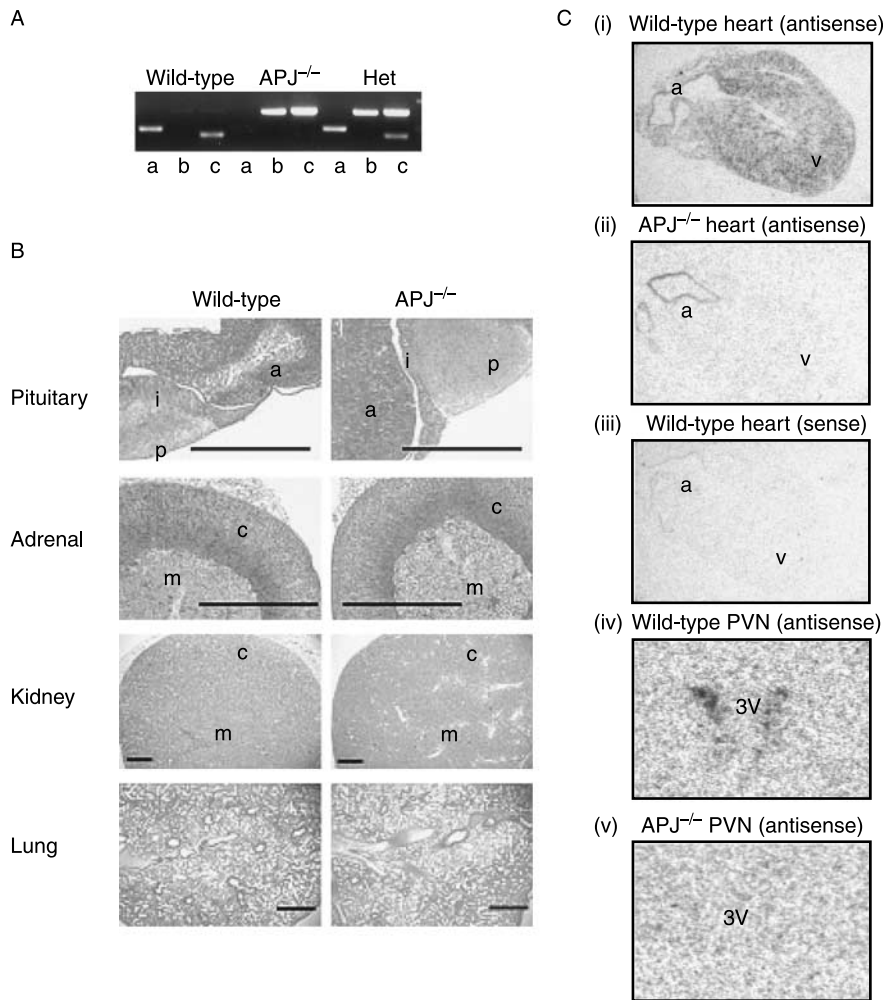


Figure 1 (A) Representative PCR of DNA extracted from tail clips from weaned pups. Image shows wild-type, heterozygous (het), and APJ knockout ($^{-/-}$) genotypes. DNA was isolated from tail tips and three reactions carried out for each sample: a, primers 1 and 2 to detect endogenous APJ; b, primers 3 and 4 to detect recombinant allele; and c, primers 5, 6, and 7 a multiplex reaction to detect both endogenous and targeted genes. (B) Comparable histology (H&E) in sections of pituitary gland, adrenal gland, kidney, and lung of wild-type and APJ $^{-/-}$ mice. Scale bars = 500 μ m; a, anterior pituitary; c, cortex; i, intermediate lobe of pituitary; m, medulla; p, posterior pituitary. (C) Representative ISHH autoradiographic images showing APJ mRNA expression in sections of heart and PVN from wild-type ((i) and (iv)) and APJ $^{-/-}$ ((ii) and (v)) mice. No APJ mRNA expression was observed in the APJ $^{-/-}$ mice. No specific labeling was seen in the wild-type control heart section using a 35 S-labeled sense-strand RNA control probe (iii). a, atria; v, ventricle; 3V, third ventricle.

Effect of water deprivation on gene expression within the HNS of wild-type and APJ $^{-/-}$ mice

To determine the effect of WD on the regulation of HNS gene expression, ISHH was performed with specific riboprobes to detect changes in *c-fos* and AVP gene expression within the MnPO, SFO, SON, and PVN, all major components of the HNS. In WR mice, low levels of *c-fos* mRNA expression were found in all regions studied that were statistically comparable between genotypes.

WD significantly increased *c-fos* mRNA expression in all regions, and to a similar extent in the MnPO (wild-type $528.9 \pm 73.1\%$; APJ $^{-/-}$ $395.2 \pm 43.7\%$) and SON (wild-type $270.8 \pm 32.2\%$; APJ $^{-/-}$ $349.7 \pm 24.4\%$) of both genotypes. However, *c-fos* mRNA expression was significantly attenuated in the SFO and accentuated in the PVN of APJ $^{-/-}$ mice versus wild-type (SFO, APJ $^{-/-}$ $377.8 \pm 61.1\%$ versus wild-type $981.3 \pm 193.7\%$ $P < 0.001$; PVN, APJ $^{-/-}$ $254.6 \pm 14.2\%$ versus wild-type $186.5 \pm 18.0\%$ $P < 0.05$; Fig. 5).

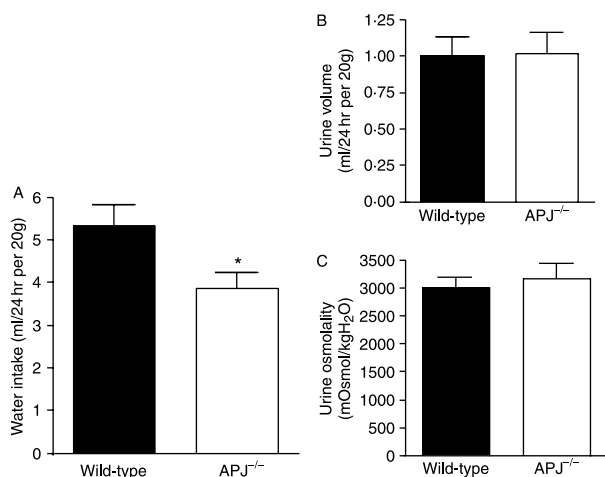


Figure 2 Fluid homeostasis in water replete wild-type (filled bars) and APJ^{-/-} (open bars) mice. Drinking behaviour (A) was significantly attenuated in APJ^{-/-} mice compared with wild-type when measured over 24 h, however, neither urine volume (B) nor urine osmolality (C) differed between the genotypes. Data are mean \pm S.E.M., $n=16$, results of t -test * $P<0.05$.

AVP mRNA levels in the SON of wild-type and APJ^{-/-} WR mice did not differ, but significantly higher levels were found in the PVN of APJ^{-/-} mice (APJ^{-/-} $127.3 \pm 7.4\%$ versus wild-type $100.0 \pm 10.3\%$; t -test $P<0.05$, Fig. 6). WD for 24 h increased levels comparably in both groups in the SON, but only in the wild-type PVN. The already elevated AVP mRNA levels in the PVN of APJ^{-/-} mice did not respond further to the dehydration stimulus.

Discussion

This is the first study to use APJ-deficient mice to determine the role of the apelinergic system in fluid homeostasis. We have shown abnormal water metabolism in APJ^{-/-} mice, manifested by a change in drinking behaviour and in the ability to concentrate urine. Under normal conditions, drinking behaviour was significantly reduced in mice lacking APJ, while other indices of fluid homeostasis, i.e. voided urine volume and urine osmolality remained unaltered. In addition, our results demonstrate that during periods of osmotic stress induced by dehydration the expected water deprivation-associated decrease in spontaneously voided urine volume and increase in urine osmolality did not occur in APJ^{-/-} mice. This defect did not appear to be due to altered regulation of AVP secretion into the circulation but may involve disrupted CVO and/or hypothalamic neuroendocrine expression as well as impaired AVP receptor-activated processes in the kidney in APJ^{-/-} mice. This study demonstrates a major physiological role for APJ in the regulation of water intake and fluid retention.

APJ^{-/-} mice were bred from heterozygous crosses and there were no apparent morphological differences in major organs of the KO mouse compared with wild-type littermates.

These findings are in accordance with other reports of comparable histology between wild-type and APJ^{-/-} mice and are consistent with reports of comparable systolic blood pressure and heart rate in APJ^{-/-} and wild-type mice (Ishida *et al.* 2004). APJ^{-/-} mice, however, showed a variable phenotype with a loss of homozygous mutants. Surviving APJ^{-/-} mice had a significantly lower body weight than wild-type littermates that may reflect the diuretic phenotype of these mutant mice, but showed no other obviously visible phenotype. Reduced body weight in APJ^{-/-} mice is also consistent with a report of chronic i.c.v. apelin administration stimulating weight gain (Valle *et al.* 2008), but apelin's precise role in feeding behaviour remains to be resolved. Partial lethality has been previously observed in KO mouse models of other GPCRs expressed in the hypothalamus and/or pituitary, such as the corticosterone releasing hormone receptor 1 (Smith *et al.* 1998, Timpl *et al.* 1998). The cause of this lethality is as yet unknown and work is ongoing within our group to explain the variable phenotype.

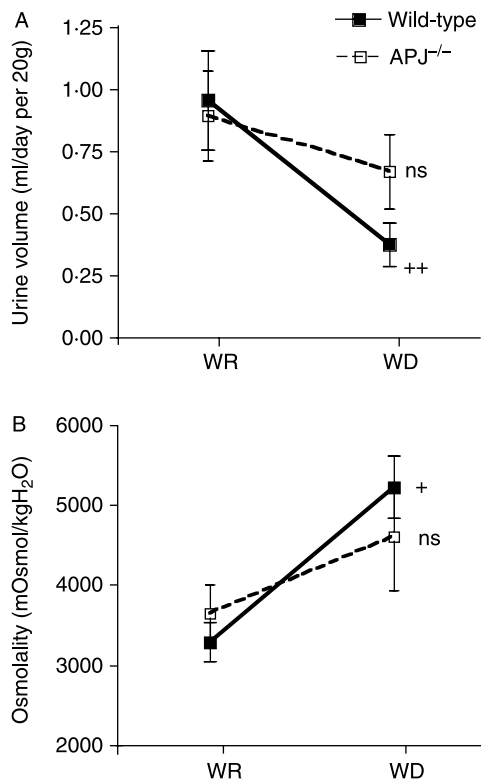


Figure 3 Effects of water deprivation on urine volume and urine osmolality. Mice were housed in metabolic cages and urine volume collected after 24 h with water (WR) followed by 24 h without water (WD). (A) WD significantly decreased spontaneously voided urine volume in wild-type (solid line) but not APJ^{-/-} (dotted line) mice compared with WR. (B) WD significantly increased urine osmolality in wild-type (solid line) but not APJ^{-/-} (dotted line) mice. Data are mean \pm S.E.M., $n=8$, results of two-way repeated measures ANOVA with Bonferroni *post hoc* test, ns, not significant; * $P<0.05$, ** $P<0.01$ WD versus WR.

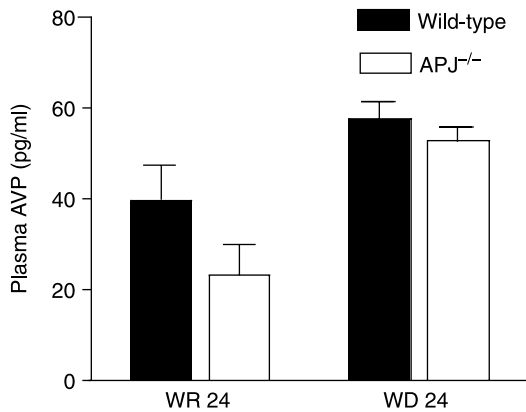


Figure 4 Plasma arginine vasopressin (AVP) concentration in wild-type (filled bars) and APJ^{-/-} (open bars) water replete (WR 24) mice and water deprived (WD 24) for 24 h. Data are mean \pm S.E.M., $n=6-8$, results of two-way ANOVA show significant comparable increases in both genotypes following water deprivation ($P<0.001$).

Our previous work suggested that aspects of the apelinergic system present in the hypothalamus where the apelinergic system is involved in fluid metabolism as dehydration was shown to be associated with a significant increase in APJ mRNA expression in the SON and PVN (O'Carroll & Lolait 2003). Specifically, involvement with the vasopressinergic system has been proposed (De Mota *et al.* 2004, Reaux-Le Goazigo *et al.* 2004, Azizi *et al.* 2008). The first apelin/APJ localisation studies provided clues that apelin may regulate vasopressinergic neurones activity. Apelin and APJ mRNA and protein are all highly expressed in the PVN and SON (Lee *et al.* 2000, O'Carroll *et al.* 2000, Reaux *et al.* 2002), and APJ mRNA and apelin protein are co-localised with AVP in both these nuclei (Reaux *et al.* 2001, O'Carroll & Lolait 2003, Reaux-Le Goazigo *et al.* 2004). In addition, apelin immunoreactivity is found along the hypothalamo-neurohypophysial tract, in the internal zone of the median eminence and in the posterior pituitary (Reaux *et al.* 2001, Brailoiu *et al.* 2002) suggesting an involvement in the control of water balance. Effective fluid homeostasis requires the rapid secretion of AVP from the posterior pituitary into the general circulation in response to various signals, including elevated plasma osmolality, decreased extracellular fluid volume and increased AngII concentration (Antunes-Rodrigues *et al.* 2004). In this study, we have shown plasma AVP levels tended to be lower in APJ^{-/-} mice than in wild-type controls under normal conditions, consistent with a recent study showing activation of AVP-neurones in the SON following administration of apelin directly onto the exposed ventral glial lamina of virgin female rats (Tobin *et al.* 2008). A dissociation between the excitatory effect of apelin on AVP cell body activity *in vivo* (implying increased AVP secretion from the pituitary) and its inhibitory effect on somatodendritic AVP release from isolated rat SON *in vitro* was shown, indicating differential regulation of AVP release by apelin (Tobin *et al.* 2008). However, conflicting results have been shown in

lactating rats (De Mota *et al.* 2004) where an inverse relationship between plasma apelin and AVP concentrations was also reported. These effects may be dependent on the physiological conditions of the animals, as AVP levels are known to be elevated in lactating animals (Poulain *et al.* 1977), or on the ongoing electrical activity of vasopressinergic cells (Tobin *et al.* 2008).

We also observed a decrease in water intake by APJ^{-/-} mice in this study, consistent with the lower plasma AVP levels found in these mice and compatible with findings from other groups of increased drinking behaviour following apelin administration in the rat (Lee *et al.* 2000, Taheri *et al.* 2002), but contrary to reports of apelin-13 inhibition of dehydration-induced water intake (Reaux *et al.* 2001) and of unaltered drinking behaviour in another APJ^{-/-} mouse line (Ishida *et al.* 2004). Inconsistent findings with regard to drinking behaviour may be due to differences in models used, species or strain of rat (Lee *et al.* 2000, Reaux *et al.* 2001, Taheri *et al.* 2002, Ishida *et al.* 2004, Mitra *et al.* 2006, Clarke *et al.* 2009). The contrasting findings on the role of the apelinergic system in drinking behaviour may also reflect the activity at distinct populations of APJ, for example i.c.v. apelin may manifest a direct effect at central APJ populations whereas peripheral administration may modulate different functions that are dependent on regional tissue site expression.

We then investigated the role of APJ in the regulation of water balance during water deprivation when endogenous levels of AVP are increased. Neurones in the SON respond electrophysiologically, functionally, and morphologically to osmotic perturbations (Dyball & Pountney 1973, Hatton *et al.* 1978, Bourque & Renaud 1984), and magnocellular neuroendocrine cells in this region produce the majority

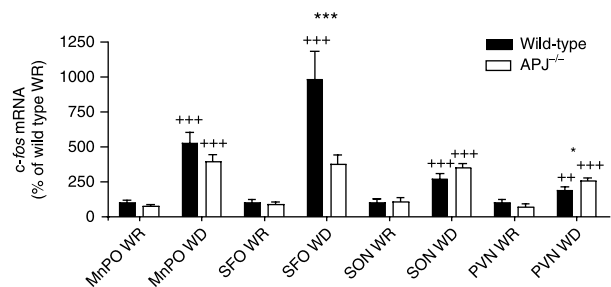


Figure 5 Effect of water deprivation (WD) on expression of *c-fos* mRNA in the MnPO, SFO, SON, and PVN. Expression of *c-fos* mRNA was negligible in water replete (WR) wild-type (filled bars) and APJ^{-/-} (open bars) mice. WD caused comparable increases in *c-fos* mRNA in the MnPO and SON of wild-type and APJ^{-/-} mice, however, the WD-induced significant increase in *c-fos* mRNA expression was significantly reduced in the SFO of APJ^{-/-} mice compared with wild-type, while in the PVN *c-fos* expression was accentuated in APJ^{-/-} mice. The graph shows expression of *c-fos* mRNA calculated as a percentage of wild-type/WR in the MnPO ($n=3$), SFO ($n=3$), SON ($n=4-5$), and PVN ($n=4-6$). Data are mean \pm S.E.M. of 2-4 images per animal and were analyzed using two-way ANOVA and Bonferroni *post hoc* tests: * $P<0.05$, *** $P<0.001$ wild-type versus APJ^{-/-}; ++ $P<0.01$, +++ $P<0.001$ WR versus WD.

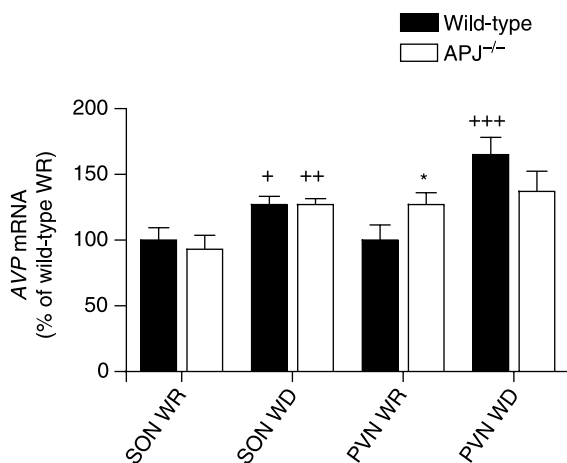


Figure 6 Expression of arginine vasopressin (AVP) mRNA in the PVN and SON of wild-type (filled bars) and APJ^{-/-} (open bars) mice housed in metabolic cages with (WR) or without (WD) free access to drinking water for 24 h. The graph shows expression of AVP mRNA calculated as a percentage of wild-type/WR in the SON ($n=4-5$) and PVN ($n=4$). Data are mean \pm s.e.m. of 2–4 images per animal, result of *t*-test to compare basal AVP mRNA expression * $P<0.05$; comparisons between genotypes and treatment groups were made using two-way ANOVA and Bonferroni *post hoc* test, + $P<0.05$, ++ $P<0.01$, +++ $P<0.001$ WR versus WD.

of AVP secreted into the systemic circulation (Antunes-Rodrigues *et al.* 2004). Hyperosmotic and hypovolemic stimulation increase the secretion of AVP and decrease peptide content and immunoreactivity in the SON and PVN (De Mota *et al.* 2004, Reaux-Le Goazigo *et al.* 2004). We show that while water deprivation significantly decreased urine volume and increased urine osmolality in wild-type controls, APJ^{-/-} mice were unable to concentrate their urine to the same extent. This defect in water homeostasis did not appear to be due to an inability of these animals to increase plasma AVP levels as comparable increases were seen in both genotypes following water deprivation, suggesting the presence of an intact central AVP system in APJ^{-/-} mice. Thus, factors other than central interactions between the apelinergic and vasopressinergic systems may contribute to apelinergic regulation of fluid homeostasis. However, our data contrast with other studies in which an increase in diuresis is seen after apelin administration to lactating female rats (De Mota *et al.* 2004, Hus-Citharel *et al.* 2008). As with drinking behaviour, these differences may be due to the physiological conditions of the animals.

As the defect in water metabolism seen in APJ^{-/-} mice did not appear to be due to altered AVP neurosecretory function, we investigated the possibility of a defect in renal mechanisms in the mutant mice. Pronounced APJ and apelin gene expression occur within peripheral tissues as well as within the CNS. Both apelin and APJ mRNA have previously been detected in the rat kidney associated with glomeruli in the cortex, in cells of the vasa recta in the inner stripe of the outer medulla, along the nephron including

the collecting ducts and in glomerular arterioles (Hosoya *et al.* 2000, Lee *et al.* 2000, O'Carroll *et al.* 2000, Kawamata *et al.* 2001, Medhurst *et al.* 2003, Hus-Citharel *et al.* 2008). Using RT-PCR, apelin and APJ mRNA have also been identified in mouse kidney (Medhurst *et al.* 2003). The strong expression of APJ mRNA in the kidney suggests an involvement of peripheral aspects of the apelinergic system in the regulation of fluid homeostasis. To determine whether the defect observed in APJ^{-/-} mice may be due to a role of APJ in regulating renal V2 receptors, the renal responses of APJ^{-/-} mice to the AVP V2 receptor agonist dDAVP were examined. Peripheral administration of dDAVP induced an increase in urine osmolality indicative of increased fluid retention in APJ^{-/-} mice; however, this increase was not as great as that seen in wild-type mice indicating an attenuation of V2 receptor-associated pathways in APJ^{-/-} mice.

In the HNS, the physiological effects of apelin appear to be mediated via AVP, perhaps by a direct action on APJ-containing vasopressinergic PVN neurones (O'Carroll & Lolait 2003). To determine whether central APJ receptors are essential for AVP neuronal function, we investigated the neurosecretory system under basal and osmotically stimulated conditions. Increased expression of the immediate early gene *c-fos* can be used as a marker of neuronal activation to identify central nuclei involved in mediating stress responses (Wilson *et al.* 2002) and was used in this study, alongside AVP mRNA levels, as an index of activity in regions involved in the central regulation of water homeostasis including the PVN, SON, MnPO, and SFO. AVP release from the magnocellular neurones during periods of osmotic stress is under the control of various aspects of the lamina terminals (McKinley *et al.* 2004). Neurones originating in the SFO, MnPO, and OVLT have direct efferent connections to the magnocellular SON and PVN, in addition there is a high degree of interconnectivity between these regions forming a complex osmoregulatory unit (Bourque *et al.* 1994). As expected, low levels of *c-fos* mRNA expression were seen in the SON, PVN, MnPO, and SFO of both wild-type and APJ^{-/-} WR mice. Neuronal activation, using dehydration as a stimulus, was seen as an increase in *c-fos* mRNA levels in all regions. However, this response was attenuated in the SFO of APJ^{-/-} mice, suggesting that APJ is required for dehydration-induced signaling in this key structure involved in the regulation of body fluid balance. An accentuated *c-fos* response was seen in the PVN of APJ^{-/-} mice, indicative of increased activity compared with wild-type. However, in this instance it was not possible to distinguish whether this specific increase originated from magnocellular or parvocellular neurones so it remains unclear as to the extent that this change might be connected to fluid metabolism. Interestingly, vasopressinergic activity within the PVN was affected in APJ^{-/-} mice as AVP mRNA levels in the PVN of these mice were elevated under basal conditions but failed to respond to the dehydration stimulus implying disruption of vasopressinergic systems in the absence of APJ. APJ activity in the SFO, PVN, and other brain regions may also be influenced either directly or

indirectly by afferent inputs – which may include the effects of apelin/APJ acting in an autocrine, paracrine, or neuromodulatory manner.

We observed that under normal conditions with free access to drinking water, APJ^{-/-} mice drank less than, but excreted comparable amounts of urine as, wild-type controls. As plasma AVP levels tended to be lower in WR APJ^{-/-} mice than in controls, it is tempting to speculate that removal of APJ has created a modified adipsic diabetes insipidus-like phenotype, a condition characterised clinically by the failure to generate the sensation of thirst in conjunction with hypotonic polyuria due to AVP deficiency (Crowley *et al.* 2007). Loss of thirst leads to failure to respond to diuresis with appropriate fluid intake and to the development of hypernatremia (Crowley *et al.* 2007). Although not possible to measure in this study due to small plasma volumes, it would be interesting to determine plasma osmolality and sodium concentrations in the APJ^{-/-} mice. Recently, apelin has been identified in adipocytes and pancreatic islets and implicated in insulin-sensitive pathophysiology, while plasma apelin concentrations have been found to be reduced in newly diagnosed type 2 diabetes mellitus (Boucher *et al.* 2005, Erdem *et al.* 2008).

In addition to involvement with vasopressinergic systems, apelin and APJ have also been linked to the renin–angiotensin system. In another APJ-deficient model, APJ^{-/-} mice displayed an enhanced vasopressor response to systemic AngII, suggesting a counter-regulatory action of apelin on AngII (Ishida *et al.* 2004). Additionally, apelin has been reported to regulate glomerular hemodynamic function in the rat kidney (Hus-Citharel *et al.* 2008). Circulating AngII activates the HNS and stimulates thirst and fluid retention via receptors located in the CVOs and subsequent release of AVP (Antunes-Rodrigues *et al.* 2004, McKinley *et al.* 2004). Mice lacking the AngII AT1a^{-/-} receptor have increased water intake, increased urine output, and decreased urine osmolality compared with controls under WR conditions and exhibit a deficit in their ability to concentrate urine in response to water deprivation (Oliverio *et al.* 2000). Increased activity within the PVN, measured by both *c-fos*-immunoreactivity and AVP mRNA expression, in AT1a^{-/-} mice following water deprivation further highlights the complexity of fluid metabolism (Morris *et al.* 2001). Despite the SFO and MnPO being key sites of AngII activity during fluid metabolism and AVP secretion there was no significant effect on cellular activity within these regions in AT1a^{-/-} mice (Morris *et al.* 2001). Emerging data thus indicate opposing roles for the apelinergic and renin–angiotensin systems (Hus-Citharel *et al.* 2008).

In conclusion, our study is an important step towards defining the physiological role of APJ in neurophysiological function using APJ deficient mice. We have shown abnormal water metabolism in APJ^{-/-} mice, possibly as a result of altered central neuroendocrine expression and disruption of the central AVP response to osmotic stimulus, as well as impaired AVP receptor-activated processes in the kidney.

Our data demonstrate a physiological role for APJ in central and peripheral mechanisms of water intake and fluid retention and suggest a novel anti-diuretic effect of apelin *in vivo*. While a targeted approach for gene knockdown would possibly circumvent the confounding effects found in global KO models, suitable tissue- or brain-region-specific Cre lines that would enable construction of relevant conditional APJ-KOs are not available and there is a paucity of APJ-selective ligands with which to explore the physiological role of APJ. The APJ^{-/-} mice should provide a useful model to investigate the role of apelin in other aspects of hypothalamic function.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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