Congenital vasopressin deficiency and acute and chronic opiate effects on hypothalamo-pituitary–adrenal axis activity in Brattleboro rats

Ágnes Domokos, Zsuzsa Mergl, István Barna, Gábor B Makara and Dóra Zelena

Institute of Experimental Medicine, Hungarian Academy of Sciences, Szigony 43, 1083 Budapest, Hungary

(Correspondence should be addressed to D Zelena; Email: zelena@koki.hu)

Abstract

A growing body of evidence suggests that vasopressinergic activity in the hypothalamus is important in stress-related behaviors (like drug abuse) in line with a role in the regulation of the hypothalamo–pituitary–adrenal axis (HPA). We hypothesized that in the naturally vasopressin-deficient Brattleboro rat, acute and chronic morphine treatment may lead to reduced HPA axis activity. Rats were treated either with a single dose of morphine (10 mg/kg subcutaneously) and serial blood samples were taken or were treated twice daily with increasing doses of morphine (10–100 mg/kg subcutaneously) for 16 days and animals were killed by decapitation 4 or 16 h after the last injection. Single morphine injection induced a biphasic ACTH and corticosterone

elevation with smaller increases in vasopressin-deficient rats. Chronic morphine treatment induced the typical somatic and HPA axis changes of chronic stress; the absence of vasopressin did not prevent these changes. In rats repeatedly treated with morphine plasma, ACTH and corticosterone levels were elevated both 4 and 16 h after the last injection (short and long withdrawal) and the absence of vasopressin attenuated this response. Our data suggest that vasopressin plays a prominent role in morphine treatment and withdrawal-induced acute hormonal changes, but does not affect development of chronic hyperactivity of the HPA axis.

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Introduction

Drug abuse is an important problem worldwide. Morphine, a drug often used for medical purposes (perioperative analgesia and cancer-related pain), is a commonly abused drug. Their connection with stress is bi-directional: exposure to stress may be related to the use of recreational drugs (Antelman *et al.* 1980, Deroche *et al.* 1992, Breese *et al.* 2004) and the use and/or more likely the withdrawal of these drugs may induce severe stress symptoms. Stress activates the hypothalamo–pituitary–adrenal (HPA) axis, a fundamental mechanism of adaptation and survival strategies. Exogenous (e.g., morphine) as well as endogenous opioids (β -endorphine and enkephalins) are believed to play an important although complex role in the control of the HPA axis. In rats, acute administration of both morphine and its antagonist, naloxone leads to HPA axis activation (e.g., Briggs & Munson 1955, Buckingham & Cooper 1986a). The activation results in a sequence of events: enhanced secretion of corticotropin-releasing hormone (CRH) from the parvocellular cells of the nucleus paraventricularis hypothalami (PVN) into the portal vessels of hypophysis, followed by increased adrenocorticotropin (ACTH) production from its precursor proopiomelanocortin (POMC) in the pituitary gland, and elevated synthesis and release of glucocorticoids (in rodents mainly corticosterone, in humans cortisol) into the general

circulation from the adrenal gland. Opiate-induced HPA axis changes appear to be mediated centrally, since some agonist stimulated *in vitro* CRH release from the hypothalamus but not the release of ACTH from the pituitary gland (Buckingham 1982, Buckingham & Cooper 1986b). Chronic administration of morphine may result in tolerance to the stimulatory effect of the opioid on the HPA axis (Buckingham & Cooper 1984, Ignar & Kuhn 1990); the secretion of pituitary ACTH in response to (other) stressors may become suppressed (Briggs & Munson 1955, Buckingham & Cooper 1984). In contrast, chronic intermittent (e.g., twice daily) administration of morphine may lead to chronic stress probably as a consequence of repeated withdrawal (Houshyar *et al.* 2001a, 2003, Zelena *et al.* 2005).

In the mammalian brain the nonapeptide, arginine vasopressin (AVP) is predominantly synthesized in magnocellular neurons located within the PVN and supraoptic nuclei of the hypothalamus and acts in the periphery as antidiuretic hormone. In addition to, but independently of, this peripheral secretion AVP is synthesized in parvocellular neurons of the PVN and together with CRH influences the release of ACTH in the anterior lobe of the pituitary gland (Antoni 1993). The role of AVP in HPA axis regulation becomes more pronounced during chronic hyperactivity of the axis (Dallman 1993, Aguilera 1994). Besides expression in the hypothalamus, AVP is also synthesized in several structures

of the limbic system (e.g., amygdala), where it affects interneuronal communication (Tribollet *et al.* 1988).

To characterize the role of AVP in physiological processes, the genetically AVP-deficient Brattleboro rat provides a good model. The AVP prohormone is processed into AVP, neurophysin, and a glycoprotein; these processes are disrupted in Brattleboro rats due to a single nucleotide deletion in the neurophysin region of the AVP gene resulting in functional AVP deficiency (Schmale & Richter 1984). Here, we studied the role of AVP in the HPA activation during acute and repeated morphine treatment in AVP-deficient Brattleboro rats. The hypothesis was that when AVP is involved in acute morphine treatment-induced HPA axis regulation, its absence will result in a less pronounced stress hormone response. We also hypothesized that AVP contributes to the development of chronic stress symptoms (Dallman 1993, Aguilera 1994) and thus a lack of AVP could reduce somatic and HPA axis changes due to chronic morphine treatment.

Materials and Methods

Animals

Male Brattleboro rats aged 3–4 months (~300 g) were bred in our institute from a colony originating from Harlan (Indianapolis, IN, USA). We compared homozygous (di/di) AVP-deficient rats with congenital diabetes insipidus with heterozygous (di/+) control rats from the same litters. The parent Long Evans strain does not seem to be an appropriate control as they were separated almost 50 years ago and therefore they could differ in other genes (Bohus & de Wied 1998, Zelena *et al.* 2003b). Moreover, the genotypes of the mother may influence the results and therefore we preferred to compare littermates (Zelena *et al.* 2003a). Rats were maintained in a controlled environment (temperature $23 \pm 1^\circ\text{C}$, humidity 50–70%, day:night schedule of 12:12 h, with lights on at 0700 h) and were fed commercial rat chow (Charles River, Budapest, Hungary) with free access to tapwater. Due to the extremely high urine production in di/di rats, their sawdust bedding was changed daily. The animals were isolated at the beginning of experiment (individual cage size: $42.5 \times 26.6 \times 18.5$ cm, 1291 Eurostandard Type III/h). All studies were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Acute morphine treatment

Anesthesia was induced by i.p. injection of ketamine (50 mg/kg, SelBruHa Állatgyógyászati Kft, Hungary)–xylazine (20 mg/kg, Spofa, Prague, Czech Republic)–promethazine chloratum (0.2 ml/kg, EGIS, Budapest, Hungary). Two days before the experiment, an intra-atrial

Silastic catheter (medical-grade silicone tubing, ID 0.64, OD 1.2 mm, Dow, Corning, MI, USA) was implanted in the right jugular vein for blood sampling. The cannula was tunneled under the skin of the back, closed and connected to a longer extension tube at the beginning of experiment. Blood samples (0.4 ml) were collected before and at 5, 15, 30, 60, and 90 min after a single s.c. morphine injection (10 mg/kg per 2 ml saline) into ice-cold tubes with 50 μl 20% K_2EDTA ; blood taken was replaced by 0.9% NaCl. The samples were centrifuged immediately and plasma was stored at -20°C until assayed for hormone content.

Chronic morphine treatment

On the basis of previous studies (Houshyar *et al.* 2001a,b), morphine dependency was induced by s.c. injections of morphine (Morphinium chloratum Ph. Eur. 4, ICN, Budapest, Hungary) twice daily at 0700 and 1900 h for 16 consecutive days (Table 1). During the first 10 days, the dose of morphine was increased by 10 mg/kg per injection each day from 10 to 100 mg/kg per injection of morphine. The controls received 0.9% saline (0.2 ml/100 g). On the morning of decapitation (at the time of last injection), we took blood from the tail under slight restraint for <2 min, cutting it with a sharp surgical knife. Half of previously morphine-treated animals got only saline (MS group, longer withdrawal, 16 h), while the other half were treated with 100 mg/kg morphine (MM group, 4 h 'withdrawal'). As a single 100 mg/kg morphine injection was lethal to 50% of naive rats (preliminary results), we decided to leave out this control group. The animals were decapitated 4 h after the last injection, at around 0011 h. Altogether, six groups from three series ($n=18$ –22 per group) were compared except for weight changes, as we measured the weight of the animals ~24 h before decapitation to avoid additional handling stress.

From decapitated animals, organ mass (thymus and both adrenal glands) were assessed in pre-weighted tubes and blood was collected into ice-cooled tubes using EDTA as anticoagulant (7–10 ml blood to 150 μl 20 w/v EDTA) for hormone measurement by RIA. After decapitation the skull was opened and the brain removed, snap-frozen on dry ice, and stored at -70°C until CRH and AVP mRNA determination by *in situ* hybridization. The whole pituitary gland was immediately frozen on dry ice in embedding medium and handled similar to brain until POMC mRNA determination by *in situ* hybridization.

Hormone measurements

Plasma ACTH was measured by RIA on 50 μl unextracted plasma using a specific antiserum developed in the Institute of Experimental Medicine (Zelena *et al.* 1999). The intra-assay coefficient of variation was 7.2% and all samples from a single experiment were measured in the same RIA. Plasma corticosterone was measured on 10 μl unextracted plasma by RIA using a specific antiserum developed in the Institute of Experimental Medicine (Zelena *et al.* 2003a). The intra-assay

Table 1 Treatment protocol in experiment 2

	di/+ or di/di								
	Control (2 ml/kg)			Morphine (mg/kg)					
	CC			MM			MC		
	07 h	11 h	19 h	07 h	11 h	19 h	07 h	11 h	19 h
Day									
1	sal.		sal.	10		10	10		10
2	sal.		sal.	20		20	20		20
...	...		...	+10		+10	+10		+10
10	sal.		sal.	100		100	100		100
11	sal.		sal.	100		100	100		100
...	...		...	...		...	...		...
16	sal.		sal.	100		100	100		100
17	sal. cort.	decap.		100 cort.	decap.		sal. cort.	decap.	

CC group got s.c. saline injections each time. MM group got increasing doses of s.c. morphine. MC group was treated with increasing doses of morphine for 16 days but the last injection was saline. sal. s.c. saline injection, cort. Blood sampling was taken through tail cut at the time when the last injection was done for plasma corticosterone measurement. decap. Decapitation 4 h after the last injection.

coefficient of variation was 7.5% and all samples from one experiment were measured in one RIA.

In situ hybridization

Frozen forebrain and pituitary tissues were mounted on a cryostat microtome and cut into 16 µm coronal sections. Every sixth brain section was mounted on a silanized slide, from anterior commissure to the end of the amygdala. Six pituitary sections were put on one slide.

The hybridization technique used was the one described by Simmons (1989) as detailed earlier (Zelena *et al.* 2006).

CRH mRNA

CRH mRNA levels were quantified by [³⁵S]UTP-containing riboprobes complementary to exonic sequences of the CRH gene (the plasmid containing 1.2 kb template was a generous gift of Dr K Majo, Northwestern University).

After the hybridization process, slides were exposed to imaging plates (Fujifilm, BAS-IP, MS 2340) for 72 h, and the plates were scanned by a fluorescent image analyzer (FLA 3000, Fujifilm, scanning resolution of 50 µm). Radiograms were evaluated by the use of the public domain National Institutes of Health (NIH) Image program (written by Wayne Rasband at NIH and available from the Internet by anonymous ftp from zippy.nimh.nih.gov). The boundary of the examined region was outlined, and the average grayness value was corrected by the background taken from neighboring hypothalamic tissue. Expression of mRNA was evaluated by summing up the grayness values measured over the whole extent of the PVN and amygdala centralis (CeA; integrated density, Barna *et al.* 2003).

AVP mRNA

AVP mRNA levels were quantified by [³⁵S]UTP-containing riboprobes complementary to the exon sequences of the AVP gene (the plasmid containing the 1.2 kb template was a generous gift of Dr K Majo, Northwestern University). To determine the AVP mRNA content of the PVN, the hybridized slides were dipped into Kodak NTB3 nuclear emulsion and exposed for 3 days. The microscopic images of the PVN region of the hypothalamus (Fig. 3A) were digitalized by a Sony CCD camera, and the images were stored on CD. Using the NIH Image J1.23 program, the optical density of the silver signal upon a given individual parvocellular cell was determined and the value obtained was corrected for background optical density measured next to the examined parvocellular cell in the same designated frame used for the parvocellular cell. From the PVN region of a given animal, the optical density of about 50–60 parvocellular cells was analyzed on 4–5 hybridized slides (Fig. 3A). The mean optical density of 50–60 parvocellular cells was used as index of AVP activity in the parvocellular region of the PVN in the given experimental animal.

POMC mRNA in the anterior pituitary

POMC mRNA levels were quantified by riboprobes complementary to the exon sequences of the POMC gene in the presence of [³⁵S]UTP (the plasmid containing 1.2 kb template was a generous gift from Dr J Eberwine, University of Pennsylvania). After the hybridization process, the slides were exposed to imaging plates, and the evaluation of the signal intensity was performed similarly as described above for CRH mRNA hybridization.

Statistical analysis

Serial blood samples from acute morphine experiment and repeated body weight measurements were analyzed by repeated measures ANOVA. Other data were analyzed by two-way ANOVA. Newman-Keuls test was used for *post hoc* comparison. All statistical analyses were performed by Statistica 6.0 statistical software (StatSoft Inc., Tulsa, OK, USA). All data are expressed as means $\pm$ S.E.M. The level of significance was set at $P < 0.05$.

Results

Acute morphine injection (Exp. 1)

The manipulation *per se* (injection and blood sampling) did not induce any ACTH or corticosterone elevation in both genotypes as may be seen in the saline-treated groups (Fig. 1, circles). The administration of a single dose of morphine (10 mg/kg) profoundly elevated HPA axis hormone levels

(effect of treatment: $P < 0.01$) in a biphasic manner demonstrated by significant treatment $\times$ time interaction ($P < 0.01$). The first ACTH peak was at 5 min, while the second was at 60 min (approximately threefold elevation in di/+) with a nadir at 15 min. The corticosterone levels followed the ACTH changes, albeit with smaller, less obvious fluctuation; a clear nadir was present at 30 min and the corticosterone level showed a second maximum at 60 min (~ 2.2 -fold elevation in di/+) where it remained constant. The absence of AVP in di/di Brattleboro rats led to a small, yet significant reduction in the elevations of both hormones (ACTH, 2.4-fold increase in di/di; effect of genotype: $P = 0.05$; corticosterone, 1.3-fold increase in di/di; genotype $\times$ time interaction: $P = 0.049$).

Chronic morphine treatment (Exp. 2)

Somatic changes The body weight of the AVP-deficient rats was smaller (initial weight di/+ : 340 ± 8 g; di/di: 290 ± 6 g) throughout the experiment (Fig. 2A; $P < 0.01$).

Figure 1 Effect of single morphine injection (10 mg/kg per 2 ml saline subcutaneously) on plasma stress hormone levels in AVP-deficient Brattleboro rats ($n = 14$ –18). (A) ACTH (fmol/ml). (B) Corticosterone (pmol/ml). Handling was without effect; morphine injection induced a biphasic elevation in both hormone levels with smaller changes in AVP-deficient rats. C, control, saline-treated animals; M, morphine treated rats. $^+P < 0.05$, $^{++}P < 0.01$ vs 0 min.; $^{***}P < 0.01$ versus saline treated; $^{\#}P < 0.05$, $^{##}P < 0.01$ versus respective di/+ group.

Figure 2 Somatic changes in chronically morphine-treated AVP-deficient Brattleboro rats ($n = 18$ –41). (A) The body weight (g) curve during the repeated treatment revealed that the addicted rats did not gain weight. (B) The relative weight of the thymus (mg/kg) was reduced in repeatedly morphine-treated rats. (C) Adrenal gland (mg/kg) hypertrophy was clearly visible after repeated morphine injections. The lack of AVP could not prevent any somatic changes induced by repeated morphine treatment, the thymus involution was even more pronounced. $^{++}P < 0.01$ versus initial weight; $^*P < 0.05$, $^{**}P < 0.01$ versus saline treated; $^{\#}P < 0.05$, $^{##}P < 0.01$ versus respective di/+ group.

The saline-treated animals of both genotypes gained weight normally (effect of time: $P<0.01$), while the repeated morphine treatment inhibited the weight gain (treatment $\times$ time interaction: $P<0.01$). There was no interaction between the treatment and the genotype suggesting that the body weight reduction was present in both di/+ and di/di rats to a similar extent.

As a sign of chronic stress, repeated morphine injections induced thymus involution (Fig. 2B; $P<0.01$) without genotype effect or treatment $\times$ genotype interaction. By pairwise comparison, the AVP deficiency aggravated the reduction of the thymus weight, so surely not prevented the development of this symptom.

Due to enhanced glucocorticoid synthesis adrenal gland hyperplasia was also visible in repeatedly morphine-treated rats (Fig. 2C; $P<0.01$) without any effect of genotype or genotype $\times$ treatment interaction.

Figure 3 Changes in the AVP mRNA signal above the parvocellular region of the PVN after chronic morphine treatment ($n=5-7$). (A) Representative slides dipped into emulsion. (B) AVP mRNA levels in the parvocellular part of the PVN (density). The levels were elevated 4 h after the last morphine injection only in control, di/+ rats. CC, saline treatment; MM, morphine treatment, decapitation 4 h after the last injection; MC, repeated morphine treatment, only the last injection was saline, i.e., decapitated 16 h after the last morphine injection. * $P<0.05$ versus saline treated; ## $P<0.01$ versus respective di/+ group.

Chronic HPA axis changes The AVP mRNA signal overlying parvocellular cells in the PVN (Fig. 3A) was enhanced in repeatedly morphine-treated di/+ animals 4 h after the last injection (Fig. 3B; MM group; $P=0.036$), while in the longer withdrawal group (16 h after the last injection; MC group) the elevation had disappeared. The AVP-deficient rats transcribe measurable amounts of the mutated AVP mRNA, but its regulation failed, and thus it was not changed at any studied time point (effect of genotype: $P=0.016$; treatment $\times$ genotype interaction: $P=0.036$).

The intensity of the CRH mRNA signal above the whole PVN was gradually increased in repeatedly morphine-injected groups with a significant rise 16 h after the last treatment (Fig. 4A; MC group; $P=0.048$). Higher elevation was induced by the AVP deficiency itself (effect of genotype: $P=0.02$). In this genotype the morphine treatment did not induce further changes, however, statistically the treatment $\times$ genotype interaction was not significant ($P=0.083$).

There was a tendency for higher CRH mRNA level in the amygdala 16 h after the last injection (MC group; $P=0.1$), however, a more robust elevation was induced by the AVP deficiency itself (Fig. 4B; $P=0.02$). The repeated morphine injections reduced the CRH mRNA level in the amygdala of di/di rats to the same extent as it was visible in di/+ rats (treatment $\times$ genotype interaction: $P=0.01$).

Repeated morphine injection significantly elevated the POMC mRNA level in the anterior lobe of the pituitary measurable at both 4 h (MM group) and 16 h (MC group) after the last injection (Fig. 4C; $P<0.01$). Both the basal and stressed levels were the same in control and AVP-deficient animals.

The resting corticosterone level of di/+ rats was elevated by the time the last injection was expected (Fig. 4D; $P<0.01$). However, the saline-treated di/di group had already higher resting levels (effect of genotype: $P<0.01$), repeated morphine treatment induced a further rise (no treatment $\times$ genotype interaction).

Plasma hormone levels after acute 'withdrawal'

There was no more than a tendency for ACTH plasma levels to be higher 4 h after the last morphine injection (MM group; $P=0.19$), but a highly significant increase was observed 16 h after the last morphine injection (Fig. 5A, MC group, eightfold increase; $P<0.01$). In AVP-deficient rats the elevation was still present however to a lesser extent (fourfold increase; treatment $\times$ genotype interaction: $P=0.059$).

Plasma corticosterone levels were significantly elevated already at 4 h after the last morphine injection (Fig. 5B, MM group, an approximately 3.5-fold increase; $P<0.01$). The longer 'withdrawal' (MC group, 16 h after the last injection) showed a more pronounced rise (15-fold; $P<0.01$). In AVP-deficient rats no significant rise was seen at 4 h 'withdrawal' (MM group; $P=0.15$), while longer 'withdrawal' (MC group; 16 h) induced significantly smaller elevations (eightfold; effect of genotype: $P<0.01$; treatment $\times$ genotype interaction: $P<0.01$).

Figure 4 Chronic changes in the HPA axis after repeated morphine injections (A). CRH mRNA level in the PVN (integrated density; $n=5-7$). Longer withdrawal stimulated the synthesis in $di/+$ rats, while in di/di animals the basal levels were already elevated without further reaction. (B) CRH mRNA level in CeA (integrated density; $n=5-7$). The AVP deficiency elevated this level but the chronic morphine treatment was able to counteract its effect. (C) POMC mRNA levels in the anterior lobe of the pituitary (arbitrary unit; $n=10-16$). Both shorter and longer 'withdrawal' induced a significant rise without any influence of the AVP deficiency. (D) Resting corticosterone levels measured from tail blood at the time last injection expected (pmol/ml; $n=19-40$). The levels were elevated by repeated morphine treatment but the AVP deficiency *per se* induced a similar rise with further increase after morphine treatment. CC, saline treatment; MM, morphine treatment, decapitation 4 h after the last injection; MC, repeated morphine treatment, only the last injection was saline, i.e., decapitated 16 h after the last morphine injection. * $P<0.05$ versus saline treated; # $P<0.05$ versus respective $di/+$ group.

Discussion

Repeated short periods of morphine withdrawal were associated with chronic activation of the HPA axis and increased the HPA axis activity at all three levels of the axis as it was already partly showed (Houshyar *et al.* 2001b, 2003, Zelena *et al.* 2005). Despite the predicted importance of AVP in chronic stress-induced HPA activity (Dallman 1993, Aguilera 1994), its absence in the Brattleboro rat did not prevent chronic stress-induced changes. On the other hand, the acute morphine treatment and withdrawal-induced hormone rises were smaller in these rats lacking AVP.

The ability of single administration of morphine to stimulate the HPA axis in rodents is well known (e.g., Buckingham & Cooper 1984). In our hands, the single subcutaneous application of a moderate dose of morphine (10 mg/kg) induced biphasic elevation of ACTH and corticosterone plasma levels. We think that this biphasic reaction was due to different stimulatory aspects of the injection, namely, the first reaction is due to the pain induced by morphine solution, while the second peak demonstrates pharmacological effect of morphine. We were able to demonstrate that s.c. morphine injection has prolonged effect when compared with i.p. injection, while the ACTH rise was

Figure 5 Plasma hormone levels due to acute 'withdrawal' at the end of repeated morphine treatment (A). ACTH (fmol/ml) levels were significantly elevated 16 h after the last morphine injection with a smaller rise in di/di rats ($n=18-23$). (B) Corticosterone (pmol/ml) levels were elevated both 4 and 16 h after the last injection with smaller rises in AVP-deficient rats ($n=18-23$). CC, saline treatment; MM, morphine treatment, decapitation 4 h after the last injection; MC, repeated morphine treatment, only the last injection was saline, i.e., decapitated 16 h after the last morphine injection. ** $P<0.01$ versus saline treated; # $P<0.05$ versus respective di/+ group; ++ $P<0.05$; +++ $P<0.01$ versus MM group.

visible at 60 min. When compared with the similar rise at 5–25 min after an i.p. injection (el Daly 1996). The absence of AVP led to lower stress-hormone levels throughout the whole examination period suggesting that AVP is important but not

an exclusive mediator of the opiate-induced HPA axis stimulation. It is worth mentioning that an elevation of the oxytocin levels in di/di rats could – at least partly – compensate its HPA axis stimulatory role as oxytocin may act on pituitary V1b receptors to stimulate ACTH release (Schlosser *et al.* 1994).

The role of AVP in opiate-induced acute HPA axis changes was supported further at the end of our chronic studies where we tested the withdrawal-induced hormone changes rather than the effect of repeated morphine administration (Buckingham & Cooper 1984). The lack of AVP diminished the 'withdrawal'-induced hormone rises in the same manner as in our acute morphine injection studies.

It appears contradictory that AVP-deficient Brattleboro rats transcribe measurable amount of AVP mRNA in their parvocellular PVN cells. It is well known that the homozygous Brattleboro rat transcribes a mutant AVP mRNA from the gene and that its expression is attenuated during development (Van Tol *et al.* 1986). The sequence of our AVP riboprobe allowed hybridization to the mutated gene. It turned out that the level of the mutated AVP mRNA in the parvocellular PVN is normal although not regulated in di/di animals. In control di/+ rats, the amount of AVP mRNA was elevated after shorter (4 h) morphine withdrawal but not at later time point (16 h), which is in accordance with our other finding about the role of AVP in short and not in long-term HPA axis regulation. Our findings are supported by the work of Houshyard *et al.* (2003), who found no AVP mRNA changes in the PVN after prolonged intermittent morphine administration and also by the work of Zhou *et al.* (2007), who demonstrated that the AVP mRNA levels in the amygdala are elevated only after early heroin withdrawal.

Consistent with the assumption that CRH mRNA in the PVN is often elevated in chronic stress (Aguilera 1994), we detected increased levels in morphine-dependent control (di/+) rats 16 h after the last injection. The shorter period of 'withdrawal' (4 h) did not induce significant changes (Lightman & Young 1988) suggesting that CRH has a regulatory role in prolonged rather than acute HPA axis changes. As the AVP-deficient rats had elevated basal CRH mRNA levels – probably due to a compensatory mechanism – we cannot conclude that the lack of elevation in this genotype was due to a regulatory role of AVP or resting levels was already at maximum and further elevation was not possible.

The action of opioids on HPA axis activity has been postulated to involve extra-hypothalamic mediators (Coventry *et al.* 2001). The central nucleus of the amygdala (CeA) may be an important site during opioid withdrawal as it is rich in cells producing the opioid and AVP system (Zhou *et al.* 2007) and a functional interaction between the opioidergic and CRHergic systems has also been postulated (Maldonado *et al.* 1992). In contrast, we could not find a significant CRH mRNA elevation in the central nucleus of amygdala of morphine-treated rats (similarly to Houshyard *et al.* 2003). On the other hand, we could demonstrate the inhibitory opioid tone on the amygdala

CRH expression (McNally & Akil 2002) in AVP-deficient rats with elevated resting levels.

Gene expression of POMC, the precursor of ACTH in the anterior pituitary, increases slowly after sustained stimulation, and thus it can reflect long-term changes as it did in morphine (Holtt & Haarmann 1985) or heroin (Zhou *et al.* 2007) dependent rats. In our hand, both the 4 and 16 h withdrawal induces similar elevations and the AVP deficiency was unable to influence the basal as well as the stressed levels.

The synthesis of glucocorticoids may be enhanced after repeated stimulation. Consequently, adrenal cortex hypertrophy, elevated resting plasma corticosterone level, and thymus involution together with body weight reduction appear the best parameters of chronic stress (e.g., Zelena *et al.* 2005) as shown here in our study with morphine-dependent rats. Thus, we could demonstrate that intermittent morphine treatment induced a chronic stress-like state (Houshyard *et al.* 2003, Zelena *et al.* 2005). However, we have to reject one of our hypotheses as AVP deficiency did not moderate the morphine dependence-induced changes. The di/di rats are smaller and likely to be chronically stressed even among basal conditions and it could prevent further development of chronic stress symptoms. In contrast, the lack of AVP even aggravated the thymus involution.

Our data do suggest that AVP plays a prominent role in acute morphine treatment and withdrawal-induced hormone changes without affecting the development of the chronic hyperactivity of the HPA axis, thus its role in the development of dependence is questionable; it may be more important in withdrawal-induced symptoms.

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