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Vasopressin deficiency diminishes acute and long-term consequences of maternal deprivation in male rat pups

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Short title: Long term consequences of maternal deprivation in Brattleboro rats

Abstract

Early life events have special importance in the development as postnatal environmental alterations may permanently affect the lifetime vulnerability to diseases. For the interpretation of the long-term consequences it is important to understand the immediate effects. As the role of vasopressin in hypothalamic-pituitary-adrenal axis regulation as well as in affective disorders seem to be important we addressed the question whether the congenital lack of vasopressin will modify the stress reactivity of the pups and will influence the later consequences of single 24h maternal deprivation (MD) on both stress-reactivity and stress-related behavioral changes.

Vasopressin-producing (di/+) and deficient (di/di) Brattleboro rat were used. In 10-day-old pups MD induced a remarkable corticosterone rise in both genotypes without adrenocorticotropin (ACTH) increase in di/di rats. Studying the later consequences at around weaning (25-35-day-old rats) we found somatic and hormonal alterations (body weight reduction, dysregulation of the stress axis) which were not that obvious in di/di rats. The more anxious state of MD rats was not detectable in di/di rats both at weaning and in adulthood (7-12-week-old).

The lack of vasopressin abolished all chronic stress and anxiety-like tendencies both at weaning and in adulthood probably as a consequence of reduced ACTH rise immediately after MD in pups. This finding suggests that postnatal stress-induced ACTH rise may have long-term developmental consequences.

Keywords: male Brattleboro pup, CRH, ACTH, corticosterone, stress-related behavior

1. Introduction

The hypothalamic-pituitary-adrenal axis (HPA) plays an important role in homeostasis. Sensitivity of the HPA axis to stress varies both during ontogeny and between individuals, the latter variance being probably due to different neonatal and early postnatal environments (Meaney et al., 1993). Early life events have been associated with profound consequences on later development (e.g. (Faravelli et al., 1986)). These environmental alterations, which permanently affect the adult phenotype and lifetime vulnerability to diseases (especially to affective disorders like anxiety and depression) are stressors and are monitored presumably by the hypothalamo-pituitary-adrenal axis (HPA) (Chrousos, 1997).

At least two hypothalamic peptides, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are important in regulating the HPA axis in adult rats. Both can reach the anterior pituitary and stimulate adrenocorticotropin (ACTH) release in a synergistic manner (Rivier and Vale, 1983, Rivier et al., 1984, Holmes et al., 1986, Raff, 1993). The relative contributions of CRH and AVP to ACTH release during stress is stressor-specific (Scott and Dinan, 1998), but there is an emerging view that in adult rats CRH serves mainly to impose stimulatory tone (Antoni, 1993, Kovacs et al., 2000).

Much less is known about the regulation of the HPA axis during development despite the importance of the perinatal period. In rats the HPA axis is already functional in late gestation (Palkovits and Mitro, 1968). From postnatal day 4 to 14, called “stress hyporesponsive period” (SHRP) (Levine, 2001), the response of the HPA-axis to stressful stimuli is markedly reduced (Sapolsky and Meaney, 1986). This period seems to be critical for the development of the animals (Sapolsky and Meaney, 1986). During SHRP the adrenal is relatively insensitive to ACTH and the levels of the primary ACTH secretagogues (CRH and AVP) are very low (Rundle and Funder, 1988, Grino et al., 1989a,b). Indeed, the regulation of hypothalamic CRH gene expression is not yet mature during the SHRP, whereas the regulation of hypothalamic AVP gene expression matures very early (Grino et al., 1989a,b). Previous works suggested that during the postnatal period AVP is the main

regulator of the HPA axis (Muret et al., 1992, Avishai-Eliner et al., 1995, Levine, 2002) in contrast to adult, where CRH seems to be the most important secretagogue (de Kloet and Oitzl, 2003).

We investigated the role of AVP in the HPA axis regulation during development using the congenital AVP deficient Brattleboro rats. The Brattleboro strain appeared in the middle of the 60`s by a spontaneous single nucleotide deletion in the neurophysin II region of AVP precursor molecule of a Long Evans rat (Sawyer et al., 1964), arresting AVP synthesis in the rough endoplasmatic reticulum. These animals suffer from diabetes insipidus because of lack of functional AVP (Evans et al., 2000). Using these animals in an earlier study we provided evidence for an important the role of AVP in the ACTH secretion during the postnatal period (Zelena et al., 2008).

In the present study we examined the long term consequences of single 24h maternal deprivation (MD) on postnatal day 10, a stimulus commonly used to assess depressive-like behaviours in adult rodents with high construct and predictive validity (Vetulani, 2013). Previous reports have shown that MD is able to induce long-term disturbances in HPA axis regulation (Suchecki and Tufik, 1997) and enhances the vulnerability to subsequent stressors (Stewart et al., 2004), although little was known about the behavioral consequences. For the interpretation of the long-term consequences it is important to evaluate the immediate effects as well. To study the contribution of AVP Brattleboro rats were used throughout (Fig.1). First, MD-induced immediate HPA axis changes (CRH mRNA level in the paraventricular hypothalamic nucleus (PVN), proopiomelanocortin (POMC, as ACTH precursor) in the anterior pituitary, ACTH and corticosterone plasma levels) were studied in pups both from primiparous and multiparous mothers. Second, postnatally MD pups were tested after weaning (25-35-day-old) for anxiety (elevated plus maze, EPM and open field, OF) and depression-like (forced swim, FS and anhedonia) behavior, as well as for HPA axis changes. Considering the importance of the amygdala (Amano et al., 2011) and its CRH content (Schulkin, 2006) in emotions CRH mRNA level in the amygdala was also studied. Third, former MD pups were tested at a later time point (young adults, 7-12 weeks of age) for anxiety- (EPM) or depression-like (FS) behavior and HPA axis changes.

2. Material and Methods

2. 1. Animals

Brattleboro rats were maintained in our Institute in a colony started from breeder rats from Harlan, Indianapolis, IN. USA. Rats were kept in controlled environment (23±1°C, 50-70 % humidity, 12 h light starting at 0700 h) and given commercial rat chow (Charles River, Hungary) and tap water *ad libitum*. We standardized the colony by mating heterozygous (di/+) females with homozygous AVP-deficient (di/di) male rats. At least 6 litters were used for each experiment. The morning on which the pups were found in the cage of the mother during the daily observations was considered the day of birth. As the number and gender of the pups can profoundly influence the maternal behavior (Moore and Morelli, 1979, Dimitsantos et al., 2007) and consequently the adult phenotype (Weaver, 2009), we standardized the litters by culling to 6 males. We compared the AVP deficient homozygous (di/di) rats with diabetes insipidus to heterozygous (di/+) control rats and MD and non-MD animals from the same litter. The genotype was determined in the pups by measuring AVP content in the homogenate of the neurointermediate lobe of the pituitary (by radioimmunoassay (RIA); Exp.1) or measuring the water consumption in older animals (Exp.2,3). All experiments were conducted in the morning between 0900 h-1200 h. The experiments were performed in accordance with regulations set by the European Communities Council Directive (2010/63/EU) and were supervised by the Institutional Animal Care and Use Committee.

2. 2. Experiments

2. 2. 1. Experiment 1. Immediate effect of MD

A similar experiment was previously conducted without controlling the littersize and gender by culling (Zelena et al., 2008), which we repeated here using standardized litters of 6 male pups (Fig.1). Three males were deprived from their mothers on day 9 for 24h while the other 3 remained with their mother. MD pups were kept together in a new cage without extra heating or lighting. All pups were killed by decapitation on day 10. The first experimental series was done on pups from primiparous mothers (n=12-27), while a second series was conducted on pups from multiparous mothers (n=9-18).

In the first experiment both brain and anterior lobe of the pituitary were dissected, collected on dry ice and stored in -70ºC until cutting for in situ hybridization. In both experiment blood was collected by decapitation.

Insert figure 1 around here,

2. 2. 2. Experiment 2. and 3. Late consequences of MD

The animals were handled as in Exp.1 except that after MD the pups were returned to their mother (the pups were marked by an ear-cut in at the beginning of MD) and examined around weaning (25-35–days-old, Exp 2; n=6-8) or as young adults (7-12-week-old, Exp.3; n=10-12) for behavior. At least 24h after the last test the animals were decapitated at rest, trunk blood, brain and pituitary were collected for HPA axis measurements and organs were dissected to measure their weights. In Exp. 2 stressed hormone levels were measured in a separate set of animals at the end of the open field test (n=9-15). For details see Fig.1.

2. 3. Behavioral tests

Rats were singly cages 2 days before the behavioral tests. Video recordings of the tests were analyzed by trained observers unaware of the experimental history of the rat being analyzed. Behavior was scored by a computer based event recorder (H77).

2. 3. 1. Elevated plus maze (EPM)

Each rat was brought in its home cage to the test room brightly lit with fluorescent ceiling lamps and immediately placed on the central arena of the EPM, with the head facing a closed arm. EPM was made of wood, painted dark grey and elevated 80 cm above the floor (arm length, 50 cm; arm width, 20 cm; central platform, 20x20 cm; closed arm walls height, 30 cm). Surface of maze was washed with water and dried before an animal was placed in it. Test duration was 5 min. Percentage of time spent in open arms and open/total (total: open plus closed) arm entries ratio (entry: three paws of animal in an arm) were calculated and used as measures of anxiety. Closed arm entries were considered as indicators of general locomotor activity.

2. 3. 2. Forced swim test (FS)

Test was performed in brightly lit room adjacent to animal facility. Rats were brought to the test room in their home cages and immediately tested. They were individually placed in a glass cylindrical tank 60 cm tall and 14 cm in diameter filled with tap water (21±1°C) at a height of 30 cm (Exp.2) or 45 cm (Exp.3). The animals were forced to swim for a 15 min period (pre-test) and 24h later were subjected to a 5 min swimming session (test; modified version of (Porsolt et al., 1977)). The test session was videotaped using a camera facing the water tank from a lateral position. Percentage of time, which the animal spent in typical immobile posture (floating) was calculated as an indicator of depression-like behavior. Rats were considered floating when their general activity was minimized to occasional and small movements of legs or tail necessary to keep their heads above the water. In addition, time which the animal spent swimming (making active swimming motions, more than necessary to merely keep the head above water) and struggling (intense movements of forepaws breaking the water surface, usually directed against the walls) was also calculated. After both swimming sessions the rats were removed from the tank, carefully dried by paper towels and returned to their home cages. Water in tank was changed after each animal.

2. 3. 3. Anhedonia

Sweet solution preference to dilute ethanol was measured using a two-bottle, free-choice test (24 h/day). The first bottle contained 2.5 w/v sucrose in tap water and the second bottle contained 8% ethanol v/v in 2.5 w/v sucrose (Huot et al., 2001). Sucrose was added to the alcohol to make the solution more palatable to the rat and increase consumption (Samson, 2000). This protocol was selected to dissect the genotype difference based on our previous report in lactating rats (Fodor et al., 2012), which suggested that testing sucrose-ethanol preference is better than a saccharin preference test in the Brattleboro rats. Fluid consumption was measured by subtracting the final weight of the bottle from the initial weight, and converted it to milliliters (assuming 1g = 1ml water). The percentage of sucrose solution from the total liquid ingested (sweet preference) was calculated.

2. 3. 4. Open field (OF)

Rats were transferred to the test room in their home cage and immediately placed in the middle of an open arena. The open field arena (OF) was a round wooden area (diameter 90 cm) surrounded by a

metal wall (40 cm), painted dark grey. The arena was virtually divided into 44 almost identical areas and the crossing of dividing lines was counted during the 10 minutes of the test. After the test the rats were immediately decapitated and blood was collected for hormone measurements.

2. 4. Hormone measurements

Blood was collected after decapitation on ice in tubes containing sodium-EDTA (20%) and after centrifugation (3000 rpm/min for 20 min) at -4°C the plasma was stored in -20ºC for later hormone measurement. Plasma ACTH was measured by RIA in 50µl unextracted plasma as described earlier (Zelena et al., 1999). The ACTH antibody (no. 8514) directed against the midportion of the h-$ACTH_{1-39}$ molecule was raised in rabbit in the Institute of Experimental Medicine, Hungarian Academy of Sciences (Budapest, Hungary). It is highly specific showing 0.2% cross-reaction with α-MSH and no significant cross-reaction with γ-MSH, CLIP, $ACTH_{11–24}$, $ACTH_{25–39}$, $ACTH_{1–14}$, and $ACTH_{1–19}$. The intraassay coefficients of variation were 4.7. Samples from one experiment were measured in one RIA. Plasma corticosterone was measured from 10µl unextracted plasma by a RIA using specific antiserum developed in our Institute as described earlier (Zelena et al., 2003). The corticosterone antiserum was raised in rabbits against corticosterone-carboximethyloxime bovine serum albumin. ^{125}I-labelled corticosterone-carboxymethyloxime-tyrosinemethyl ester was used as tracer. The interference with plasma transcortin was eliminated by inactivating transcortin at low pH. Assay sensitivity was 1 pmol. The intraassay coefficient of variation was 12.3. Samples from one experiment were measured in one RIA. The AVP content of pituitary extracts (Exp.1) was measured by specific RIA. The neurointermediate lobes (Exp.1A) or whole pituitaries (Exp.1B) from each animal were taken out and were put into 100µl 0.1N HCl in an Eppendorf tube and frozen on dry ice. Frozen pituitaries were placed into boiled water for 5 minutes. Tubes were removed from water and were centrifuged with 3000 rpm for 15 minutes and after that the pituitaries were homogenized by an ultrasonic homogenizer. After homogenization the samples were frozen overnight. On second day samples were thawed at room temperature (RT), centrifuged for 24 min at RT with 10000 rpm and supernatant was transferred to an empty Eppendorf tube. Samples were stored at -20 °C until hormone measurement. Anti-AVP antiserum was produced in a rabbit was a gift from Dr M. Vecsernyés

(Szent-Györgyi Med. Univ., Szeged, Hungary). The intra- and inter-assay coefficients of variation were 5.42 and 19%, respectively.

2. 5. In situ hybridisation

Rats were decapitated under basal conditions (Exp. 2-3) or at the end of MD (Exp.1) and the brain and hypophysis rapidly removed from the skull, frozen on dry ice and stored in -70°C until measurement. Brain and pituitary sections of 16µm were cut in a cryostat and hybridized as previously described (Zelena et al., 2006). The sections containing the PVN were selected with the help of a microscope and a rat brain atlas (Paxinos and Watson, 1998). After hybridization CRH mRNA (brain) and POMC mRNA (hypophysis) levels were quantified by means of ^{35}S-UTP containing riboprobes complementary to the exonic sequences of the gene (the CRH probe was obtained from Dr D. Richter, University of Hamburg, Germany, while the plasmid containing the POMC template was a generous gift of Dr J. Eberwine, University of Pennsylvania). After hybridization slides were exposed to imaging plates (Fujifilm, BAS-IP, MS 2340) for 72h (CRH) or 16h (POMC) and the plates were scanned by an image analyzer (FLA 3000, Fujifilm, scanning resolution 50µm). Radiograms were evaluated by the ImageJ program (http://rsbweb.nih.gov/ij/). The average grey value of three sections on both hemispheres (CRH) or six sections (POMC) taken at 80µm intervals was used for the analysis (Fodor et al., 2012).

2. 6. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the ANOVA/MANOVA module of the STATISTICA 11.0 software package (Tulsa, OK, USA). The factors for two-way ANOVA were MD and genotype. ANOVA assumptions were evaluated by the Levene's test. Multiple pairwise comparisons were made by the Newman-Keuls method. Data are expressed as mean±standard error of the mean (SEM) and the level of significance was set at $p<0.05$.

3. Results

Insert Table 1 around here,

3. 1. MD in 10-day-old male pups (Exp.1)

MD decreased the body weight independently of genotype ($F_{(1,30)}$=22.8, p<0.01) (Table 1.). The weight of AVP- deficient pups (di/di) was smaller by approximately 25% at this age ($F_{(1,30)}$=11.7, p<0.01) and MD induced further changes (by approximately 20%; no interaction).

In the PVN of 10-day-old pups the CRH mRNA levels were lower after MD than in non-deprived animals ($F_{(1,27)}$=8.0, p<0.01) (Fig.2A). AVP-deficiency itself also induced a reduction in CRH mRNA levels ($F_{(1,27)}$=92.6, p<0.01) independently from MD (no interaction).

MD resulted in reduction of CRH mRNA in the amygdala ($F_{(1,22)}$=42.5, p<0.01) (Fig.2B) in both genotypes. On the contrary, AVP-deficiency induced an increase of the amygdala CRH mRNA level ($F_{(1,22)}$=8.3, p<0.01), independently from MD (no interaction).

The early life time stress stimulus as well as the AVP-deficiency did not have an effect on the POMC mRNA in the anterior lobe of the pituitary (data not shown).

MD increased plasma ACTH levels ($F_{(1,74)}$=14.83, p<0.01) (Fig.2C). Surprisingly, there was no appreciable ACTH elevation in AVP-deficient pups (di/di) at the end of MD. The effect of AVP-deficiency was highly significant ($F_{(1,74)}$=10.53, p<0.01) with significant interaction between the two factors ($F_{(1,74)}$=14.53, p<0.01).

The plasma concentrations of corticosterone in 10-day-old pups increased after MD ($F_{(1,74)}$=73.75, p<0.01) (Fig.2D). In contrast to ACTH, AVP-deficiency induced an increase in the corticosterone levels ($F_{(1,74)}$=7.55, p<0.01), independently from the effect of MD.

Insert figure 2 around here,

In a separate set of animals we tested if previous maternal experience (previous labours) of the mother could influence the HPA axis reactivity of the pups (Table 2.). Similarly to the offspring of primiparous mothers we found a significant effect of MD ($F_{(1,48)}$=4.61, p<0.05), AVP-deficiency ($F_{(1,48)}$=7.07, p<0.01) and their interaction ($F_{(1,48)}$=7.26, p<0.01) on plasma ACTH levels. In case of the plasma corticosterone levels we had again very similar results to that in primiparous mothers. Namely,

both MD ($F_{(1,49)}$=57.00, p<0.01) and AVP-deficiency ($F_{(1,49)}$=7.86, p<0.01) induced significant alterations without any interactions.

Insert Table 2 around here,

3. 2. Changes at weaning (Exp.2)

3. 2. 1. Somatic parameters

Approximately 2 weeks after single MD the body weight of MD animals were still significantly smaller by approximately 10% than that of their counterparts ($F_{(1,73)}$=7.66, p<0.01) (Table 1., 3 weeks). AVP-deficient animals were smaller (by approximately 30%; $F_{(1,73)}$=118.6, p<0.01), and MD-induced body weight reduction was present also in this genotype (no interaction). During the course of experimentation the body weight gain was also reduced in MD animals ($F_{(1,28)}$=5.38, p<0.05) with smaller weight gain in di/di rats ($F_{(1,28)}$=5.12, p<0.05) (data not shown).

MD had no effect on relative organ weights (thymus, adrenal gland, spleen) (Table 3.). The relative weight of the adrenal gland was higher in AVP-deficient animals ($F_{(1,73)}$=6.64, p<0.05) without interaction with MD or effect on thymus or spleen weights.

Insert Table 3. around here,

3. 2. 2. HPA axis

At weaning previous MD induced a significant reduction in the CRH mRNA levels of the PVN ($F_{(1,23)}$=9.76, p<0.01), which was more pronounced in control than in AVP-deficient animals (interaction: $F_{(1,23)}$=7.60, p<0.05) (Fig.3A). In the amygdala similar changes were detectable (MD: $F_{(1,23)}$=4.98, p<0.05; interaction: $F_{(1,23)}$=4.61, p<0.05) (Fig.3B).

Resting ACTH levels were lower in MD than control animals ($F_{(1,25)}$=4.84, p<0.05) with a tendency of reduced levels in di/di animals ($F_{(1,25)}$=3.61, p=0.06) (Fig.3C). OF test, as a novel environment, remarkably elevated the plasma ACTH concentrations ($F_{(1,69)}$=75.1, p<0.01) (Fig 3C,E). The elevation was smaller in MD animals ($F_{(1,44)}$=3.85, p=0.05). In di/di rats with overall lower ACTH levels MD failed to further decrease stressed plasma ACTH (genotype: $F_{(1,44)}$=18.2, p<0.01; interaction: $F_{(1,44)}$=4.23, p<0.05).

Resting plasma corticosterone levels were not influenced by previous MD, but were higher in AVP-deficient rats ($F_{(1,25)}$=10.88, p<0.01) (Fig.3D). OF resulted in a significant elevation of plasma corticosterone levels without any influence of previous MD or genotype (Fig.3F).

Insert figure 3 around here,

3. 2. 3. Behavioral measures

On the EPM di/di animals were less anxious as they spent more time on the open arm ($F_{(1,26)}$=5.07, p<0.05) (Fig.4A). MD reduced the time spent in open arm in di/+ genotype, while it had an opposite effect in di/di rats (interaction: $F_{(1,26)}$=8.10, p<0.01). Similar effects were detectable in the case of locomotion independent measure of anxiety, the open arm entries (genotype: $F_{(1,26)}$=9.11, p<0.01; interaction: $F_{(1,26)}$=4.59, p<0.05) (Fig.4B). It was not surprising as the locomotor activity measured by closed arm entries as well as OF was not influenced either by MD or by the genotype (data not shown).

During the FS test di/di animals showed reduced depression-like behavior with less time spent in immobile posture (floating: $F_{(1,28)}$=8.93, p<0.01) (Fig.4C) and enhanced struggling ($F_{(1,28)}$=10.0, p<0.01; data not shown) without any influence of previous MD.

The reduced depressive-like behavior of AVP-deficient rats was further supported in an anhedonia test, where di/di rats drank more sucrose compared to the total fluid intake ($F_{(1,44)}$=15.2, p<0.01) (Fig.4D). Previous MD failed to influence this parameter.

Insert figure 4 around here,

3. 3. Changes in adulthood (Exp.3)

3. 3. 1. Somatic parameters

In adulthood (7 weeks of age) the MD-induced body weight reduction was not detectable, although the di/di animals were significantly smaller (by approximately 10%; $F_{(1,38)}$=9.2, p<0.01) (Table 1.). During the course of the experimentation the body weight gain was not influenced by previous MD, but the AVP-deficient animals gained smaller weight ($F_{(1,38)}$=21.2, p<0.01; data not shown).

MD had no effect on relative organ weights (thymus, adrenal gland, spleen) (Table 3.). The relative weight of spleen was higher in AVP-deficient animals ($F_{(1,39)}$=26.98, p<0.01) without interaction with MD or effect on thymus or spleen weights.

The food intake was influenced by MD differently in the two genotypes (interaction: $F_{(1,39)}$=5.77, p<0.05) (di/+C: 66.3±1.3g, di/+MD: 58.4±2.1g, di/diC: 64.5±2.6g, di/diMD: 66.8±2.4g). More specifically, the MD-induced food-intake reduction was not present in AVP-deficient, di/di animals.

3. 3. 2. HPA axis

Postnatal MD induced an elevation of the CRH mRNA levels in the PVN in adulthood ($F_{(1,14)}$=6.04, p<0.05) (Fig. 5A). The AVP-deficiency per se diminished the levels ($F_{(1,14)}$=34.5, p<0.01), but had no effect on MD-induced changes.

The POMC mRNA in the anterior lobe of the pituitary was elevated in MD animals ($F_{(1,40)}$=4.42, p<0.05) (Fig.5B). In contrast to CRH mRNA the AVP-deficiency elevated the POMC levels ($F_{(1,40)}$=8.09, p<0.01) without influencing the MD-induced changes.

Neither resting plasma ACTH (Fig. 5C) nor corticosterone levels (Fig.5D) were influenced by either studied factors (MD or genotype).

Insert figure 5 around here,

3. 3. 3. Behavioral measures

On the EPM there was a significant interaction between MD and genotype in case of the open arm time ($F_{(1,40)}$=5.23, p<0.05) (Fig. 6A). Namely, di/+MD rats spent less time in the open arm than non-MD rats, but not in the case of AVP-deficient animals. There was only a tendency for similar interaction in case of open arm entries ($F_{(1,41)}$=3.55, p=0.06) (Fig.6B) without any significant effect on closed arm entries (data not shown).

Insert figure 6 around here,

During the FS test we did not find any significant effects on the studied parameters (data not shown).

4. Discussion

4.1. Hormonal changes

Present results support our previous conclusion (Zelena et al., 2008, Zelena et al., 2011, Makara et al., 2012) that AVP is the main stimulator of the ACTH secretion during the postnatal period. CRH synthesis in the PVN is presumably not mature during this period as CRH mRNA levels did not respond to MD. The lower CRH mRNA level of di/di animals suggests that AVP may be necessary for the maturation of CRH positive cells of the PVN. Despite these later differences the next level of the axis seems to be unaffected as reflected by the identical POMC mRNA levels in all groups. We might assume that at this age the CRH secretion is enough to maintain a basal POMC synthesis rate, but not for increasing processing during the prolonged stress of MD. Another explanation could be that during the postnatal period CRH is not the predominant stimulatory factor for POMC and consequently ACTH synthesis.

The present results reinforce previous findings that in the early postnatal period corticosterone secretion may increase in response to stressors with little or no preceeding or parallel ACTH elevations (intermittent hypoxia: (Johnson et al., 2013, Chintamaneni et al., 2014); AVP-deficiency combined with MD (Zelena et al., 2008, Varga et al., 2011), Hypnorm injection (Zelena et al., 2008), insulin-induced hypoglycaemia (Zelena et al., 2011), ether inhalation (Makara et al., 2008, Zelena et al., 2011)). Other studies suggest that adrenocortical stimulation in the absence of ACTH rise is a general mechanism, detectable not only in pups (Johnson et al., 2013, Bodager et al., 2014), but also in adults (Bornstein et al., 2008). Thus, there may be a yet unknown ACTH-independent regulation of the corticosterone secretion (Polenov et al., 1982), e.g. through β-adrenoceptors or some other mechanisms (Makara et al., 2012). Although an ACTH elevation may not be required for adrenocortical activation ACTH may have extraadrenal roles (for a review see (Zelena and Makara, 2012)) and the lack of ACTH elevation in the di/di pups could be important for the long-term behavioral effects of the stress reaction.

Our present results do not contribute to the long debate if AVP released only from the parvocellular cells of the PVN or also AVP from magnocellular origin has a role in the HPA axis

regulation (Holmes et al., 1986, Raff, 1993, Engelmann et al., 2004). We should add, however, that in pups the distances, diffusion and permeabilities could be different and the role of magnocellular AVP may be more important than in the adult animals.

Although previous studies have demonstrated that prior reproductive experience can influence the glucocorticoid levels of the offspring either through maternal physiology or behavior (Onyango et al., 2008) in the present study parity had no similar effect.

The rat`s stress experience may be reflected in body and organ weights as established by Hans Selye (Selye, 1936). In experimental animals excessive glucocorticoid supply is accompanied by reduced body weight (Bazhan and Zelena, 2013, Iwasa et al., 2014). However, the negative metabolic consequences of MD were not detectable in adulthood (Table 1.). Moreover, the body weight decrease immediately after MD was similar in di/+ and di/di pups supporting the similarity of the stressor-intensity. The thymus is sensitive to chronic stress, reacts with atrophy and in medical literature is often referred to as "barometer of stress" (Gruver and Sempowski, 2008). Another lymphoid organ, spleen also reacts to excessive glucocorticoid supply with atrophy (Veenema et al., 2003). Since MD had no effect on the organ weights we suggest that MD does not induce long-term overall metabolic and immunological effects. Although the AVP-deficient rats are living with a mildly activated stress system as indicated by the elevated resting corticosterone levels, higher relative adrenal weight in young animals and by an enhanced POMC mRNA in adulthood, the lymphoid organs (thymus, spleen) of adult di/di animals reflects a rather normal immune status (Orzechowski et al., 2000).

The reduced CRH mRNA level of PVN in MD animals at weaning, but an elevation in later life seems to reflect ongoing changes long after postnatal stress. Although in pups the lower CRH mRNA after MD can be explained by enhanced corticosteroid negative feedback (Schmidt et al., 2005), but around weaning no resting glucocorticoid elevation was detectable in MD animals. Nevertheless, at this timepoint the lower CRH mRNA level of di/+ animals can contribute to their reduced stress-reactivity suggesting that a switch from AVP to CRH as main regulator of the HPA axis might happen around weaning. Subtle alterations in adulthood suggest that the missing influence of AVP in homozygous di/di rats should be compensated by other mechanisms (Zelena et al., 2009a).

CRH compensation is unlikely as CRH mRNA levels were decreased rather than increased in the absence of AVP not only in pups but also in adult animals (Makara et al., 2012). Because of structurally similarities oxytocin is another good candidate for compensation. In a previous study we have shown that in adult Brattleboro rats the hypothalamic oxytocin concentration is increased (Zelena et al., 2009b). Oxytocin levels in postnatal Brattleboro rats have not been measured so far, nevertheless functional restitution can be excluded as the ACTH reactivity is substantially altered in di/di pups.

4.2 Behavioral consequences

The main goal of the present studies was to study the involvement of AVP in the long-term consequences of strong postnatal stress. As the development of long-term consequences may be time dependent we studied animals both at weaning and 2 months later in adulthood.

A pathogenic role of hyperactive HPA axis in affective disorders is well established (Scott and Dinan, 2002, Frank and Landgraf, 2008). In contrast, the AVP-deficient Brattleboro rats exhibited persistent mild elevations of resting corticosterone levels but were less anxious and depressive-like in the EPM, FS and sucrose preference tests (Mlynarik et al., 2007). Consistent with this behavioral pattern around weaning they showed reduced stress-reactivity measured by smaller ACTH elevation at the end of 10 min OF. This suggests that the lack of AVP has a stronger influence on behavior than a mild, sustained elevation of plasma glucocorticoids.

Paradoxically, MD animals presented similar reduced stress-reactivity but enhanced anxiety-like behavior. Human twin studies found also blunted stress reactivity in bullied children (Ouellet-Morin et al., 2011). Nevertheless, we might assume that MD-induced changes in the brain CRH mRNA levels rather than in stress hormone levels underline the behavioral alterations. Indeed, at weaning the reduced CRH mRNA level in the amygdala region of MD animals and its enhanced level in AVP-deficient MD animals (Fig.3B) were in good agreement with similar changes in the time the animals spent in the open arm of the EPM (Fig.4A,B). On the contrary, in adulthood our previous results reported elevated CRH mRNA level in amygdala of MD rats (Barna et al., 2003). Thus, MD-induced extrahypothalamic CRH mRNA alterations are influenced not only by the timing of MD (i.e.

in 6-12-18-day-old pups (Vazquez et al., 2006)), but also by the recovery time after MD (i.e. examination in 25-35-day-old vs 7-12-week-old rats).

Our experiments might fit the Engel's biopsychosocial model (Engel, 1977) or its experimental interpretation by the three hit model (de Kloet et al., 2007). The genetic susceptibility is represented by a defective gene (neurophysin II - AVP), the postnatal environmental alteration is modeled by MD and behavioral testing represents an acute stimulus, which might exacerbate the symptoms. However, in Brattleboro rat strain a single MD was unable to make the animals vulnerable to adult psychogenic stimuli. Although we might assume that in adulthood a behavioral test as a single acute stressor is not strong enough to exacerbate anxiety- and depressive-like symptoms, but in separate experiments conducted on di/+ Brattleboro animals we established that even a chronic mild stressor paradigm applied for 5 weeks is unable to elicit a depressogenic effect of MD (Supplementary Table 1., no interaction between MD and chronic mild stress). An explanation may be in strain-dependent susceptibility differences. It has been shown that early postnatal dexamethasone treatment mimicking aversive postnatal environment was also ineffective in Long Evans (origin of Brattleboro strain) while being effective in other rat strains (Wistar or Spague Dawley) (de Kloet et al., 2014). It is also possible that the prolonged exposure to the postnatal stressor is what matters. The most popular paradigm of postnatal early-life stress is repeated maternal separation for varied periods of time from 15 min to 8 h (Maniam et al., 2014), which has been studied in both mice and rats for more than five decades. It is known to affect both the HPA axis and behavioral responses in mothers and offspring. MD (absence of the dam for a more extended period used also in our present experiment) is another common form of early-life stress which has been also studied for decades. As during MD the amygdala CRH mRNA level decreased while plasma corticosterone increased, it is reasonable to conclude that this stressor is a nutritional rather than emotional stimulus (Maniam et al., 2014). In contrast, repeated separation is a prolonged psychogenic stressor applied during a long period of development. Therefore it is not surprising that several days' intermittent maternal separation induced profound long term psychological changes, while a single MD may induce just subtle affective alterations.

5. Conclusions

AVP-deficiency counteracted some of the effects of MD. Namely, in di/di animals at weaning the MD-induced CRH mRNA reduction both in the PVN and amygdala was absent, the reduced ACTH failed to rise and the anxiogenic effect was also missing. In adulthood beside the CRH mRNA elevation and anxiogenic effect of MD the reduced food intake was also couteracted by AVP-deficiency. In view of the profound mitigating actions of AVP deficiency AVP antagonists might be useful in the treatment of anxiety disorders at the time of the unpleasant situation and may prevent the late consequences.

During the postnatal period the ACTH elevation to MD was abolished in the absence of AVP and alternative secretagogues of glucocorticoids may explain the stress-induced rise. Single MD failed to induce profound long term hormonal or behavioral changes. Nevertheless, the lack of AVP abolished long-term chronic stress and anxiety-like alterations suggesting that the prevention of postnatal ACTH elevation during the acute phase of MD could have positive effect on long-term outcome. We might conclude that ACTH, beyond its glucocorticoid releasing effect, might have other important role during development such as programming the brain for later life (Zelena and Makara, 2012).

Figure captions

Fig.1 Timeline of the experimental designs. MD: single 24 h maternal deprivation; EPM: elevated plus maze for 5 min; FS1: forced swim for 15 min; FS2: forced swim on the next day for 5 min; OF: Open field for 10 min.

Fig.2 Effect of single 24 h maternal deprivation (MD) in 10-day-old pups of primiparous mothers (Exp.1A). *In situ hybridization studies*: Panel A. CRH mRNA in the PVN was significantly smaller in MD and in AVP-deficient animals without interaction. Panel B. CRH mRNA in the amygdala decreased in MD and increased in AVP-deficient rats. *Plasma levels*: Panel C. ACTH (fmol/ml) was significantly elevated by MD only in di/+ pups. Panel D. Corticosterone (pmol/ml) increased after MD in both genotypes. n=12-27 **p<0.01 *vs.* undisturbed control; #p<0.05; ##p<0.01 *vs*. di/+ pups

Fig. 3 HPA axis changes at weaning (25-35-day-old) in previously maternally deprived (MD) AVP-deficient Brattleboro rats (Exp.2). *In situ hybridization studies:* Panel A. CRH mRNA in the PVN decreased in MD di/+ but not in di/di rats. Panel B. CRH mRNA in the amygdala showed similar changes. *Plasma levels:* Panel C. Resting ACTH (fmol/ml) levels were lower in MD rats without any effect of AVP-deficiency. Panel E. Open field for 10 min stimulated the ACTH secretion with smaller increase in MD di/+ rats. Panel D. Resting corticosterone (pmol/ml) levels were higher in AVP-deficient rats without any effect of MD. Panel F. Open field induced a rise in corticosterone levels without any influence of previous MD or genotype. n=6-15 *p<0.05, **p<0.01 *vs.* non-MD control; #p<0.05; ##p<0.01 *vs*. di/+ rats

Fig.4 Behavior in AVP-deficient Brattleboro rats maternally deprived on Day 9-10 for 24h (MD) and tested at weaning (25-35-day-old; Exp.2). Panel A. Open arm time decreased in MD di/+ animals only and increased in di/di rats. Panel B. Open arm entry frequency showed similar changes. Panel C. Forced swim test showed reduced floating time in AVP-deficient animals without any effect of MD. Panel D. The sweet preference of AVP-deficient animals was higher than that of di/+. n=5-15 *p<0.05 *vs.* non-MD control; #p<0.05; ##p<0.01 *vs*. di/+ rats

Fig. 5 HPA axis changes in adulthood (7-12-week-old; Exp.3) in previously maternally deprived (MD) AVP-deficient Brattleboro rats. *In situ hybridization studies:* Panel A. CRH mRNA in the PVN was significantly increased in MD and decreased in AVP-deficient rats without interaction of the two factors. Panel B. POMC mRNA in the anterior lobe of the pituitary was elevated by both factors (MD and AVP-deficiency) without interaction. *Plasma levels* were not influenced by MD and/or AVP deficiency*:* Panel C. Resting ACTH (fmol/ml) and Panel D. corticosterone (pmol/ml) levels. n=10-13 *p<0.05 *vs.* non-MD control; ##p<0.01 *vs.* di/+ rats

Fig. 6 Behavior on the elevated plus maze. AVP-deficient Brattleboro rats were maternally deprived on Day 9-10 for 24h (MD) and tested in adulthood (7-12-week; Exp.3). Panel A. Open arm time was decreased in MD di/+ animals only. Panel B. Open arm entry frequency showed similar tendencies. n=10-13 #p<0.05 *vs*. di/+ rats

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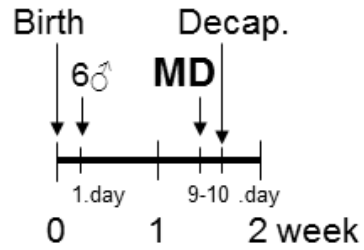
Fig.1

Exp.1



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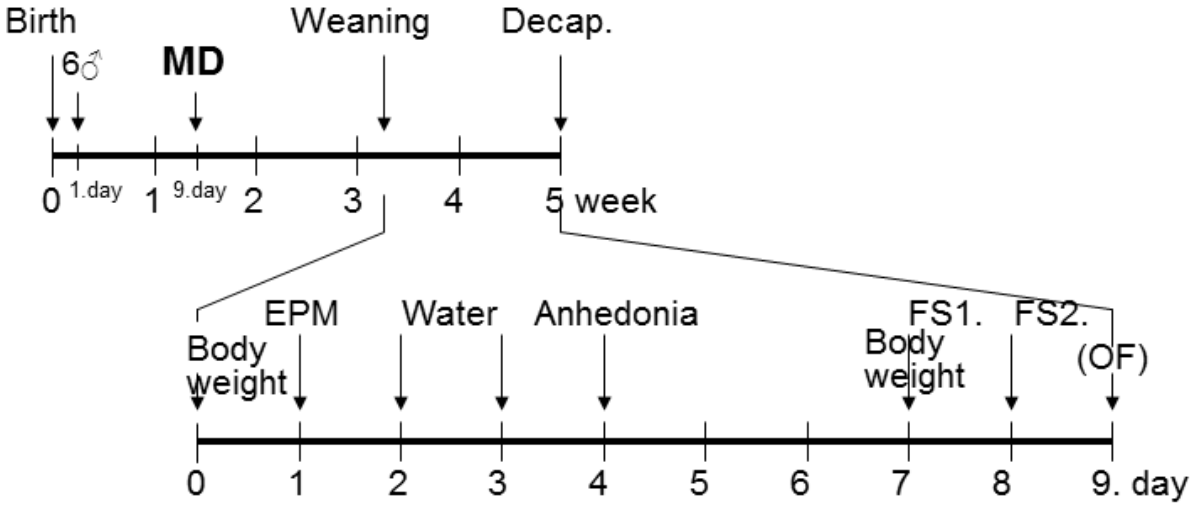

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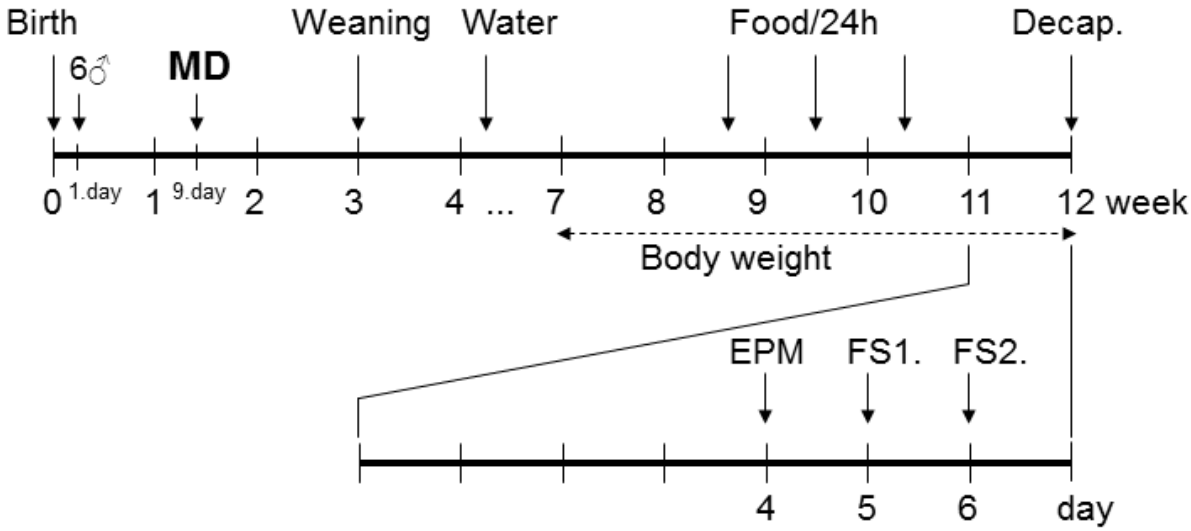

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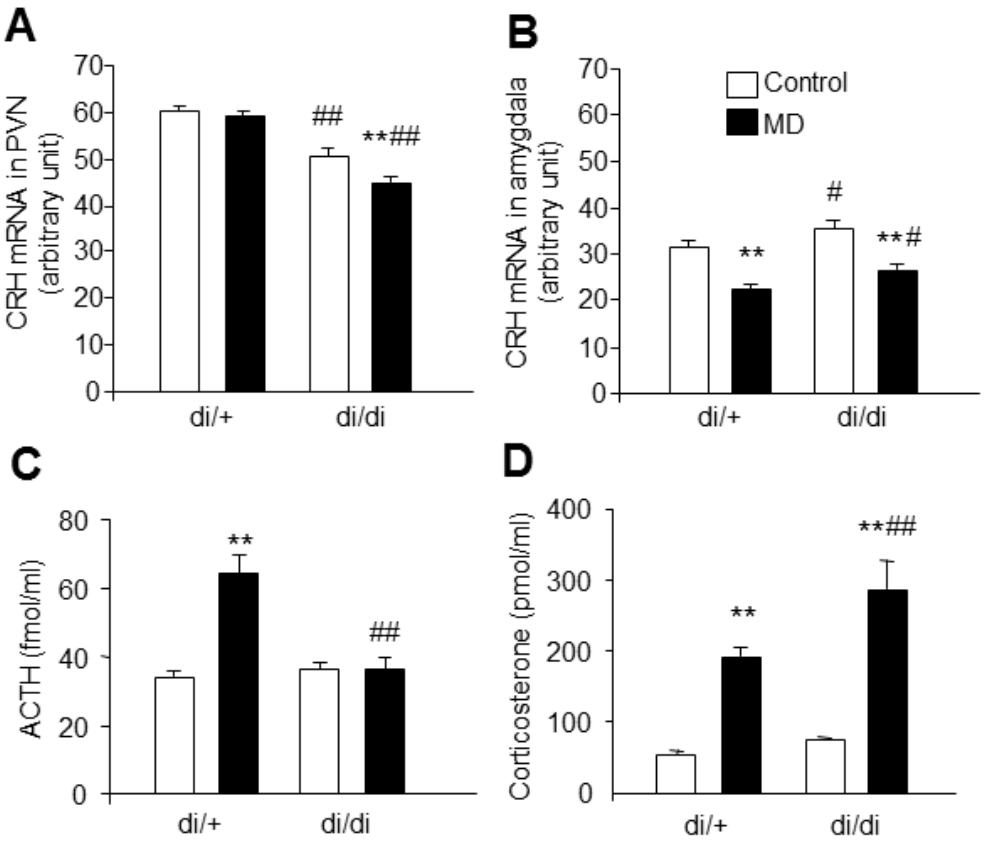

Fig.3

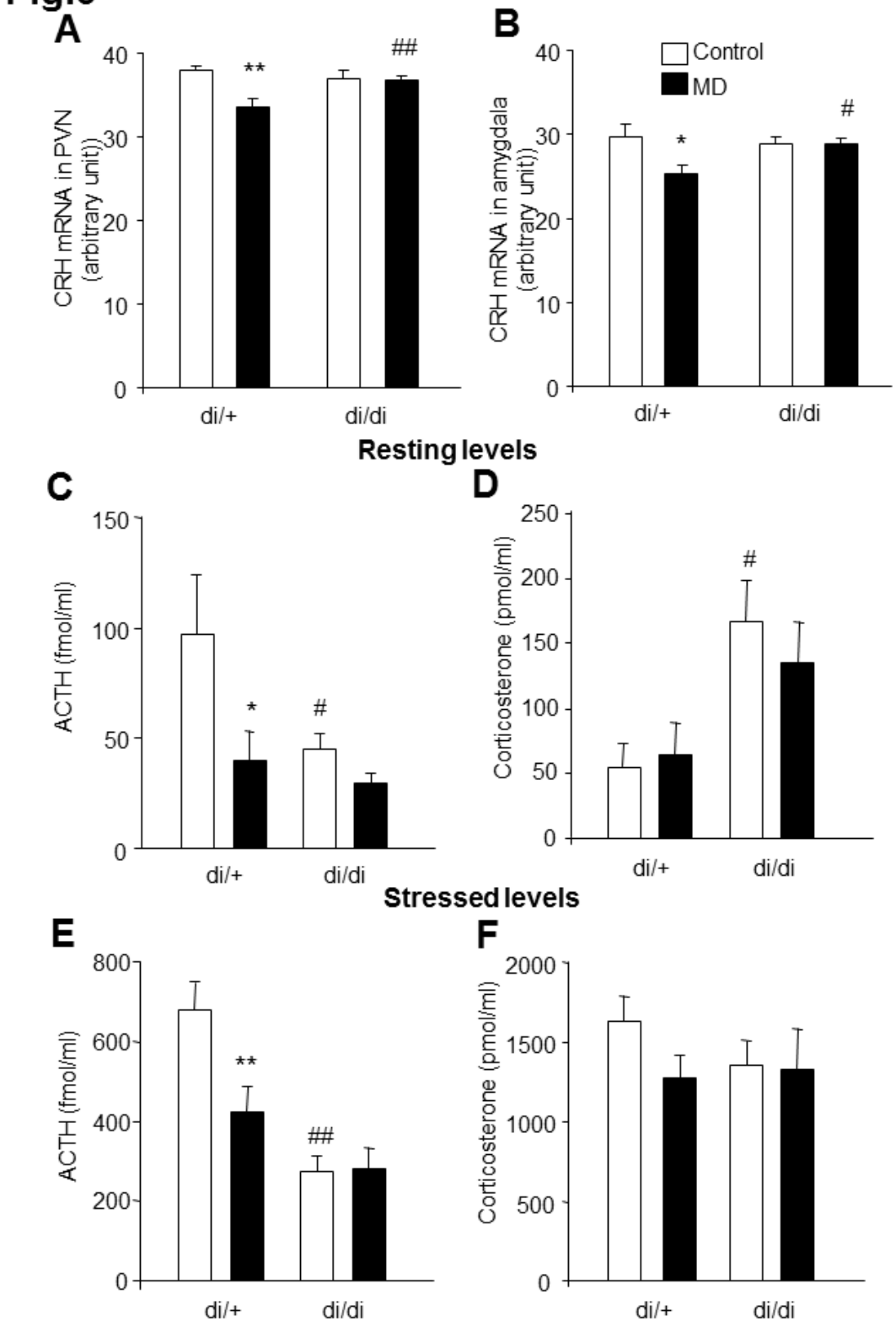

Fig.4

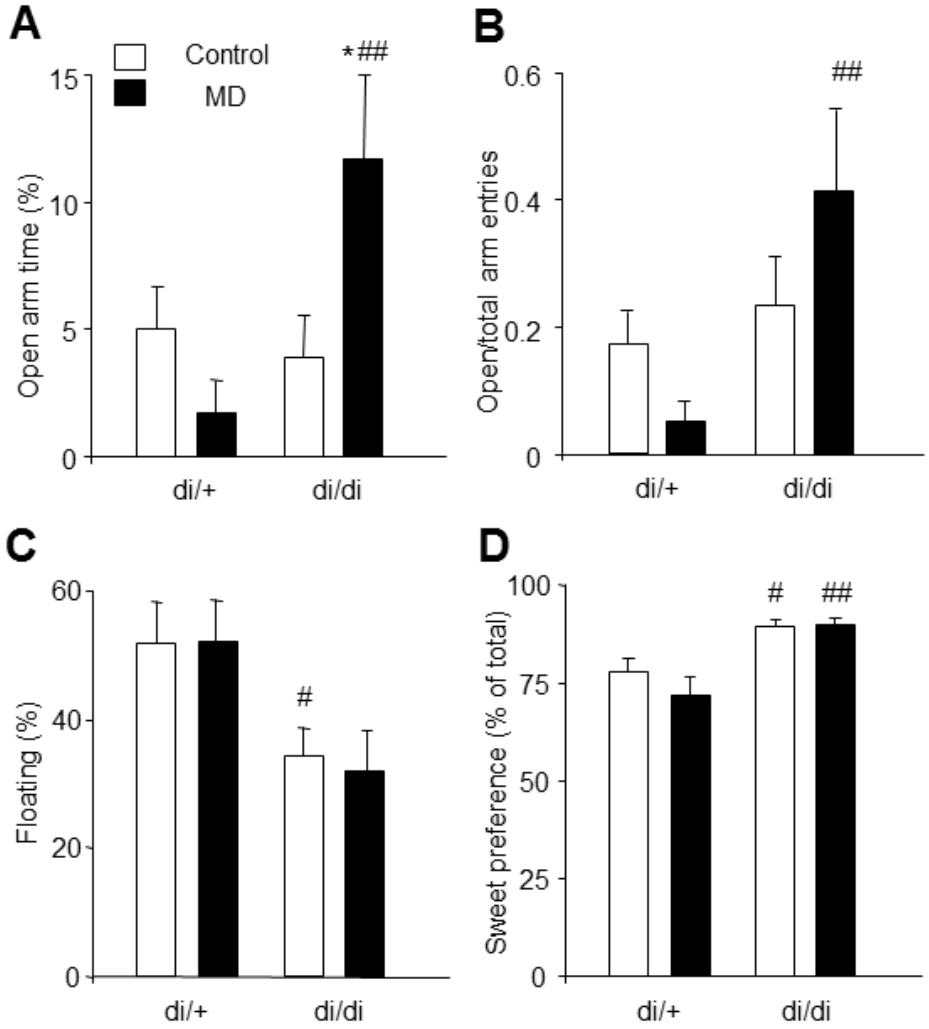

Fig.5

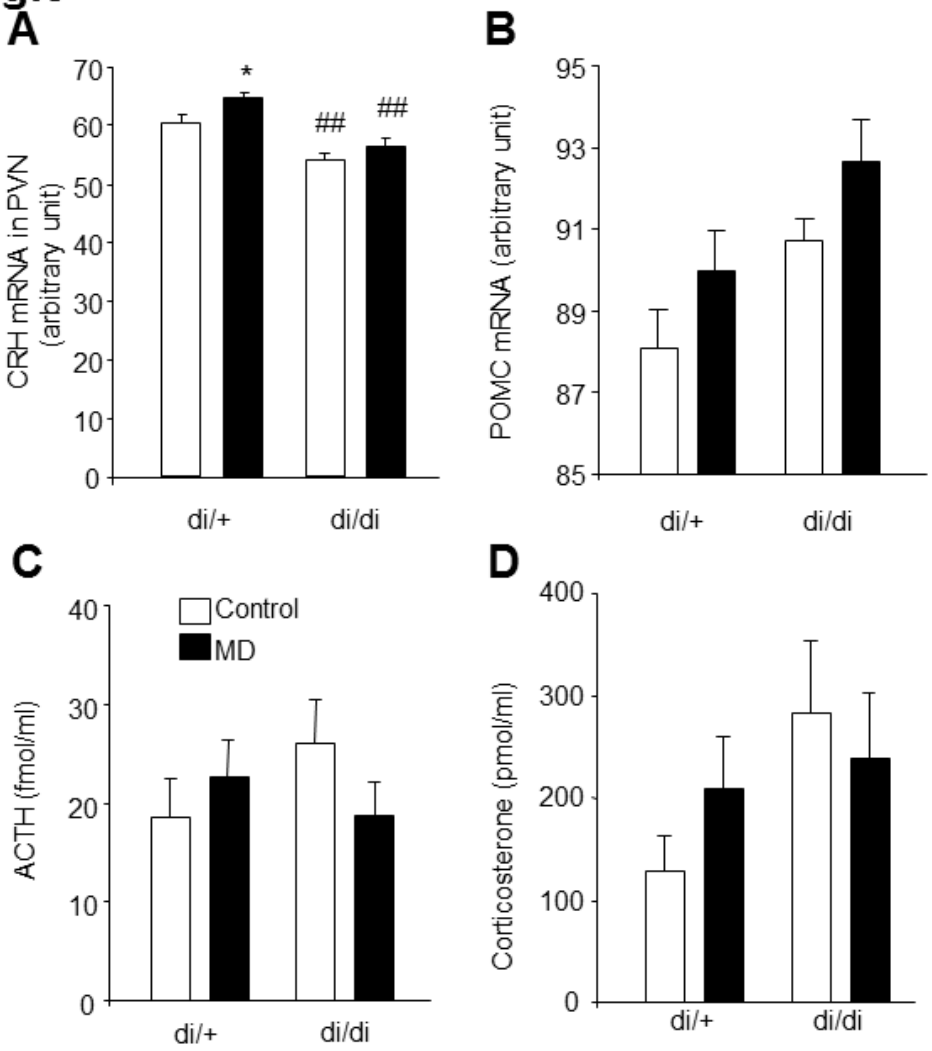

Fig.6

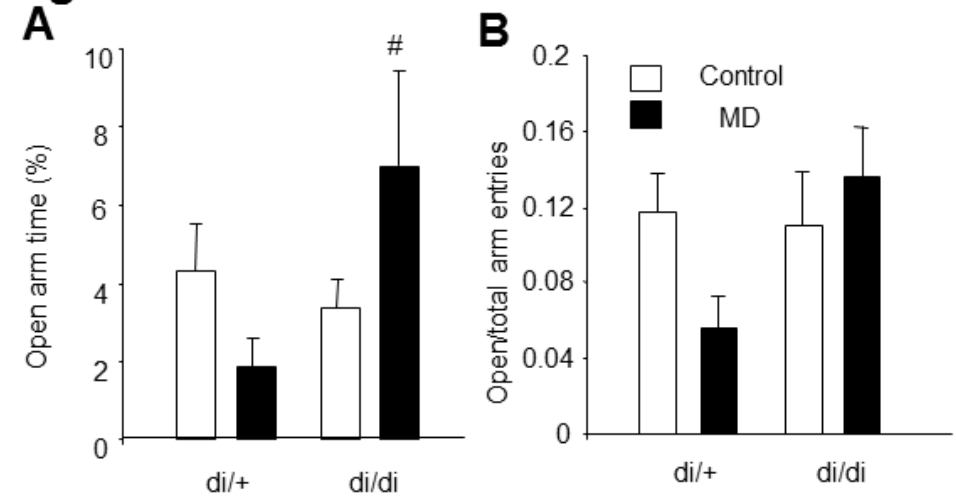

Table 1. Body weight at different ages: influence of 24h maternal deprivation at 9-day-old pups and AVP-deficiency

		10 days	3 weeks	7 weeks
di/+	Control	23.2±0.92	92.8±2.1	292.8±16.8
	MD	19.2±0.59*	84.2±3.3*	311.9±15.5
di/di	Control	17.6±1.48##	62.2±2.4##	268.0±16.9
	MD	13.5±1.29*##	55.8±2.6##	234.7±18.2#

MD: 24h maternal deprivation; *p<0.05 *vs.* control, non-MD; #p<0.05, ##p<0.01 *vs*. di/+

Table 2. Changes in plasma stress-hormone levels after 24h maternal deprivation in 10-day-old Brattleboro pups of non-primipara mothers (Exp. 1B)

		di/+		di/di	
	Number of animals	control	MD	control	MD
ACTH (fmol/ml)	18,14,11,9	33.5±4.4	61.4±8.0**	33.7±2.8	30.6±3.6##
Corticosterone (pmol/ml)	18,14,11,10	19.6±2.7	87.0±9.1**	32.1±4.3##	139.3±26.4**#

MD: 24h maternal deprivation; **p<0.01 *vs.* unseparated animals; #p<0.05; ##p<0.01 *vs*. di/+

Table 3. Organ weight changes at different ages: influence of 24h maternal deprivation (MD) in 9-day-old pups and AVP-deficiency

		di/+		di/di	
	Relative organ weight (mg/kg)	control	MD	control	MD
Weaning (Exp.2)	Thymus	4510.5±132	4652.9±119	4867.7±191	4672.0±191
	Adrenal gland	231.8±8.7	227.2±10.6	255.1±13.2	263.8±14.6
	Spleen	5038.9±294	4870.2±210	5041.6±274	5151.8±440.3
Adulthood (Exp.3)	Thymus	849.1±51.8	726.2±59.3	880.1±88.2	830.2±54.5
	Adrenal gland	95.9±4.5	95.0±6.7	91.2±7.5	89.4±6.2
	Spleen	2037.5±77	2204.2±59	2494.6±85##	2591.7±102##

##p<0.01 *vs*. di/+

Table 4. Summary of interactions between MD and AVP-deficiency

	Somatic parameters	HPA axis (ACTH)	Behavior (EPM)
pup	-	+	nm
young	-	+	+
adult	-	-	+

nm: not measured

Supplementary material
Supplementary Material

Table 1. Chronic mild stress (CMS) in maternally deprived (MD) animals (di/+)

	Control		CMS		Statistic	
	Control	MD	Control	MD	CMS	MD
Body weight change (6w)	112.3±12.0	48.6±6.6	100.5±10.6	43.5±5.2	p<0.01	N.S.
Thymus weight (mg)	341.6±21.5	265.1±11.5	303.2±20.4	257.4±14.6	p<0.01	N.S.
Weight of adrenal gland (mg)	38.3±1.8	32.8±1.1	40.2±2.5	36.7±1.9	p<0.05	N.S.
Weight of spleen (mg)	810.8±32.5	773.0±25.1	924.2±31.8	796.1±31.4	p<0.01	p<0.05
Food intake (g/kg)	65.4±1.5	53.4±2.1	59.6±2.2	54.7±2.2	p<0.01	N.S.
ACTH (fmol/ml)	18.5±3.9	33.7±5.7	22.7±3.7	23.9±2.5	p=0.053	N.S.
Corticosterone (pmol/ml)	129.1±32.9	490.5±65.6	209.6±50.4	361.5±53.4	p<0.01	N.S.
Open arm time (%)	4.3±1.19	1.6±0.78	1.9±0.76	0.8±0.31	p=0.01	p<0.05
Closed arm entries (counts)	5.8±0.84	7.8±0.95	8.4±0.80	8.1±0.96	N.S.	N.S.
Open/total arm entries	0.117±0.021	0.047±0.015	0.056±0.017	0.024±0.009	p<0.01	p=0.01
% floating	3.785±0.58	7.975±2.05	3.533±0.75	4.573±0.55	p<0.05	N.S.

There was no statistical interaction between the effect of MD and CMS in any of the studied cases. Although in the case of corticosterone the interaction was almost significant (p=0.052), despite our expectation the levels of previously MD animals were lower and not higher. For detailed description of the CMS procedure see (Varga, Domokos et al. 2011).