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ORIGINAL ARTICLE

Anxiogenic role of vasopressin during the early postnatal period: maternal separation-induced ultrasound vocalization in vasopressin-deficient Brattleboro rats

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Abstract Both animal and human studies suggest that in adulthood, plasma vasopressin level correlates well with anxiety. Little is known about the mood regulation during the perinatal period. Here, we aim to investigate the influence of vasopressin on anxiety during the early postnatal age. As a sign of distress, rat pups emit ultrasonic vocalizations (USVs) when they are separated from their mother. This USV was detected in 7- to 8-day-old vasopressin-deficient Brattleboro pups, and they were compared to their heterozygote littermates and wild-type pups. The results were confirmed by V1b antagonist treatment (SSR149415 10 mg/kg ip 30 min before test) in wild-types. Chlordiazepoxide (3 mg/kg ip 30 min before test)—an anxiolytic—was used to test the interaction with the GABAergic system. At the end of the test, stress-hormone levels were measured by radioimmunoassay. Vasopressin-deficient pups vocalized substantially less than non-deficient counterparts. Treatment with V1b antagonist resulted in similar effect. Chlordiazepoxide reduced the frequency and duration of the vocalization only in wild-types. Reduced vocalization was accompanied by smaller adrenocorticotropin levels but the level of corticosterone was variable. Our results indicate that the anxiolytic effect of vasopressin deficiency (both genetic and pharmacological) exists already during the early postnatal age. Vasopressin interacts with the GABAergic system. As mood regulation does not go parallel with glucocorticoid levels, we suggest that vasopressin might have a direct effect on special brain areas.

Keywords Ultrasound vocalization · Vasopressin · SSR149415 · Anxiety · Postnatal period · Chlordiazepoxide · GABA

Introduction

Arginine vasopressin (AVP) is a hormone that plays an important role in water homeostasis and circulation (Decaux et al. 2008). Besides, it is a key peptide in the regulation of complex social behaviors in mammals (e.g., regulation of aggression, memory, social recognition, pair bonding and parental behavior) (Caldwell et al. 2008). It is synergistic to corticotrophin-releasing hormone (CRH) on pituitary corticotroph cells activating the hypothalamic-pituitary-adrenocortical (HPA) axis (also called stress axis), which is one of the main background mechanisms of adaptation (Aguilera 1994; Selye 1975). Dysregulation of the HPA system plays a causal role in the symptomatology of anxiety disorders (stress-related illnesses) (Keck 2006; Keck and Holsboer 2001).

As the pituitary contains V1b receptor (V1bR), this was the main target of anxiety research and pharmacological development (Hodgson et al. 2014; Iijima et al. 2014). *SSR149415* was the first selective, non-peptide V1bR antagonist, with potent oral antagonist effects on corticotrophin (ACTH) secretion and anxiolytic-like properties in rodents (Serradeil-Le Gal et al. 2002). When tested in classical animal models of anxiety, such as the light/dark box, the elevated plus maze test (EPM), social interaction, and the punished drinking tests, SSR149415 produced

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anxiolytic-like effects (Amikishieva et al. 2011; Serradeil-Le Gal et al. 2005). Hypophysectomy diminished the anxiolytic effect of SSR1494154 in a social interaction test (Shimazaki et al. 2006) suggesting a strong HPA axis contribution.

Brattleboro rats are naturally occurring models of genetic AVP deficiency (Buckingham and Leach 1980; McCann et al. 1966). They have a spontaneous mutation in the neurophysin gene region (part of the AVP precursor), which leads to inappropriate structure of AVP with accumulation of abnormal AVP precursors in the membrane of the endoplasmic reticulum and Golgi apparatus, therefore, to diabetes insipidus phenotype: polyuria, polydipsia, and reduced ability to concentrate urine (Guldenaar and Pickering 1988; Krisch et al. 1986; Sausville et al. 1985; Schmale et al. 1984; Valtin and Schroeder 1964). Coherent with the suspected role of AVP (Frank and Landgraf 2008; Zelena 2012) and the effect of SSR149415, adult male AVP-deficient animals was shown to be less anxious in many laboratory tests (Mlynarik et al. 2007). Little is known about the mood regulation during the perinatal period.

Anxiety disorders are prevalent among childhood psychiatric disorders. Up to 15–20 % of the youth population has an anxiety disorder (Costello et al. 2005). It is maybe even under diagnosed, because the physical symptoms cannot be medically explained most probably accounted for anxiety or depression (Campo et al. 1999). Previous studies using Brattleboro rats, SSR149415, and antiserum suggested that during the postnatal period, AVP is the dominant regulator of the ACTH secretion (Makara et al. 2012; Muret et al. 1992; Torpy et al. 1994; Zelena et al. 2008, 2009b, 2011). Based upon the strong correlation between HPA axis and mood, one might hypothesize an important role of AVP in anxiety regulation during this early age, too, most probably through the V1bRs at the pituitary.

The possibilities to study the anxiety state of an infant are limited. One of the best tools is to examine the maternal separation-induced ultrasound vocalization (USV). This USV is typically 35–40 kHz, lasts about 0.1–3.5 s at a sound pressure of 60–80 dB (Hofer 1996; Hofer and Shair 1978; Miczek et al. 1995). USV can be produced already hours after birth, the peak occurs between post natal days 7–9 (Branchi et al. 2001), after eye-opening at day 14 becomes progressively harder to elicit, and subsequently disappears around day 18 (Allin and Banks 1971). This test has been regarded as an index of separation anxiety and is used for studying the pharmacological basis of associated disorders. Anxiolytic drugs, like benzodiazepine receptor agonists (enhancing the effect of gamma-aminobutyric acid (GABA) on GABAA receptors) (Gardner 1985; Hodgson et al. 2008; Olivier et al. 1998) or partial and full serotonin (5-HT) receptor agonists (Benton and Nastiti 1988; Hard and Engel 1988; Kehne et al. 1991; Olivier et al. 1998; Winslow and Insel 1991), reduce USV. Chlordiazepoxide hydrochloride (CDP) is the first benzodiazepine synthesized in the mid-1950 s, and it also reduces the USV (Hodgson et al. 2008). On the other hand, pentylenetetrazol, which has been reported to be clinically anxiogenic, increases the number and the power of these calls (Insel et al. 1986).

The Present study in Brattleboro rats was designed to test the hypothesis that reduced AVP is responsible for developing lower anxiety level already during early postnatal ages. We predicted that congenital deficit of AVP would lead to attenuated anxiety-like behavior in USV test during maternal separation in connection with its HPA axis regulatory role. To prove our hypothesis, we measured the stress-hormone levels (ACTH and corticosterone) right after testing and treated wild-type pups with V1bR antagonist. To explore the interaction between AVPergic and GABAergic systems, CDP, a well-known anxiolytic drug, was also applied on AVP-deficient infants.

Materials and methods

Subjects

Brattleboro rats were maintained at the Institute of Experimental Medicine in a colony started from breeder rats from Harlan, Indianapolis, IN, USA. We compared male and female 7- to 8-day-old Brattleboro pups from the same litters with mating homozygous AVP-deficient (d*i*/d*i*) fathers with heterozygous (d*i*/+) mother rats; in this way, d*i*/+ and d*i*/d*i* rats were born (Bohus and de Wied 1998; Zelena et al. 2003b). We have chosen the age based on data from the literature (Blumberg et al. 2000; Branchi et al. 2001), as well as upon preliminary results with different age groups. Separate sets of Brattleboro pups were measured in the USV test in their 3-, 5-, 7-, and 9-day old in order to determine their ultrasound production, and they had a peak on the 7th postnatal days (see Fig. 1h). The litter size was not controlled. As male and female pups did not differ in the studied parameters, we presented the pooled data from both genders. The genotype of the pups was assessed after the experiment upon the AVP content of the pituitary by radioimmunoassay. For Exp. 2. exceptionally, while for Exp. 1, additionally homozygous "wild-type" (+/+) Brattleboro rats were also used from separate families with +/+ mothers and fathers. Pups were housed with the dam in their home cage under standard laboratory conditions (temperature: 23 ± 1 °C; relative humidity: 50–70 %). The day/night schedule was 12/12 h, with lights on at 07:00 h. All experiments were done between 10:00 and 14:00.

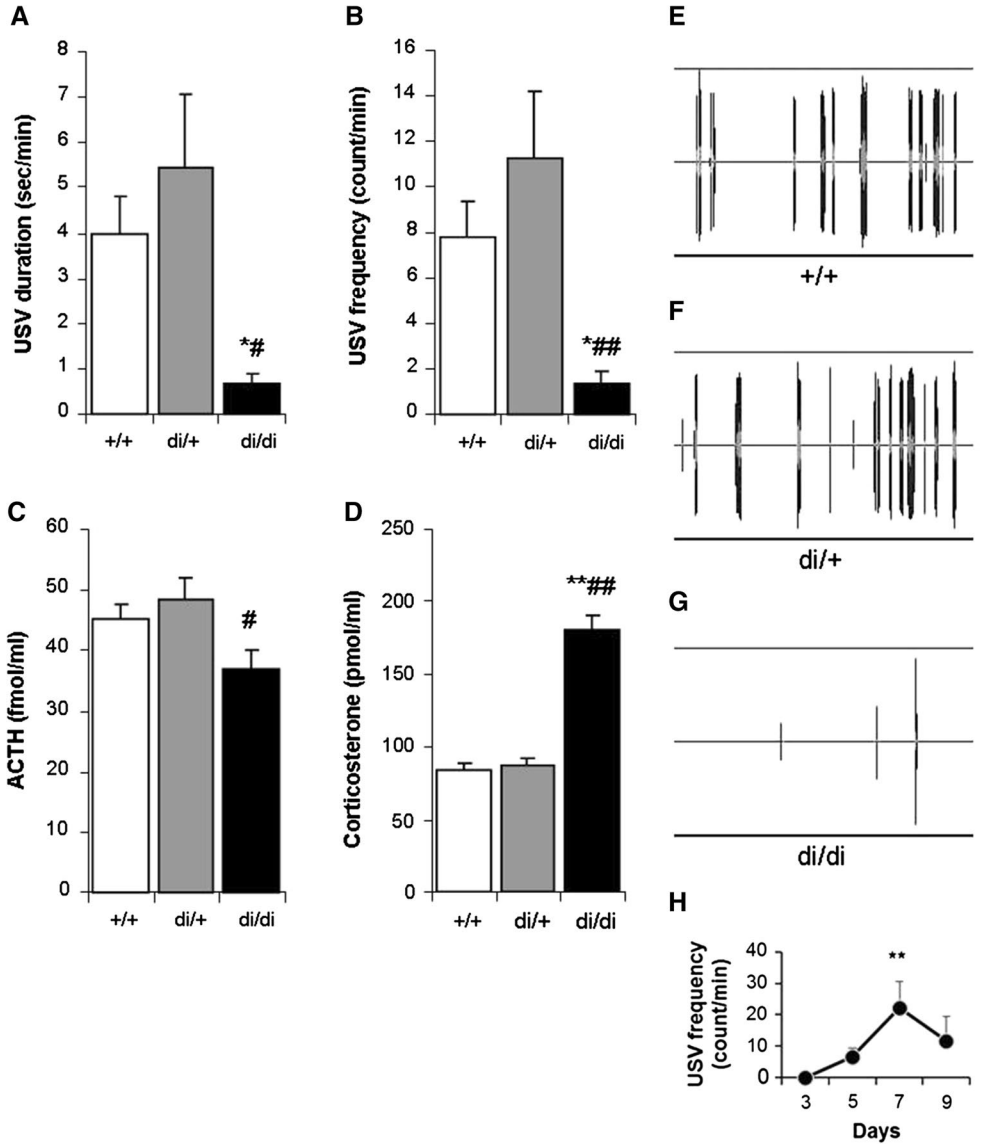

Fig. 1 Comparison of different genotypes of the Brattleboro rat strain. 7- to 8-day-old animals underwent a 10 min maternal separation-induced ultrasound vocalization (USV) test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. +/+; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. di/+

Methods

USV

On the day of the study, the rat pups and their dam were moved from the animal housing room to another room and left undisturbed for at least 1 h before initiating the study. In order to minimize maternal effects, pups from the same litter were randomly assigned to treatments. Pups from at least three different mothers were used for each experiment. In the case of pharmacological manipulations pups, one after the other in every 12 min received an intraperitoneal (ip) injection of the drug or vehicle (1 μl/g), and they were marked and returned to the dam and littermates. Thirty minutes after the administration pups were brought to a soundproof room and placed in a 2-l glass beaker without bedding and heating. USVs were followed for 10 min. Only one animal was tested at the same time. Individual calls during this period were detected using an ultrasound-sensitive frequency division detector (CIEL-electronique, CDB205 R2). It divides the original signal by 10, to transform it down. The advantage is that the whole spectrum of the detector will be converted to the hearing range at one time, so the detector serves as a microphone. Vocalizations were recorded using a free Audacity 1.2.6. software and stored on a personal computer.

In previous studies, large portions of USVs emitted by 8-day-old rats were found from 30 to 50 kHz (Allin and Banks 1971; Blumberg et al. 2000). Thus, signals were filtered and the power spectrum was analyzed ranging from 30 to 50 kHz. Data were automatically counted using a Rat Call Counter software (developed by S. Zsebők). The threshold value was set at a signal amplitude of 0.4 V to exclude background noise.

Experiment 1 Genotypes. Homozygous wild-type, homozygous AVP-deficient, and heterozygous pups were randomly examined in maternal separation-induced USV test. At the end of the 10 min examination period, the pups were decapitated. Trunk blood was collected for stress-hormone measurements, while hypophysis was collected for AVP determination to establish the genotype.

Experiment 2 V1bR antagonist treatment in wild-type (+/+) Brattleboro pups. SSR149415 (10 mg/kg, a generous gift from the Sanofi-Synthelabo company) was suspended in 0.4 % Tween 80 and was delivered ip 30 min prior to testing. Previously this dose effectively diminished the lipopolysaccharide-induced ACTH elevation in 10-day-old pups (Zelena et al. 2011). At the end of the test, the pups were decapitated, and trunk blood was collected.

Experiment 3 Chlordiazepoxide hydrochloride (3 mg/kg CDP; Sigma Chemical Co., St. Louis, MO, USA) was dissolved in saline, and the 7- to 8-day-old littermates of both genotypes (d*i*/+ and d*i*/d*i*) were injected ip 30 min before the 10 min USV test. This dose previously diminished the USV even at high temperature without influencing the locomotor activity (Olivier et al. 1998). At the end, the infants were tested for sedation by the negative geotaxis test placing them on a 45° inclined foam-rubber board with their nose pointing down. The latency until the animals rotated their body through 180° was measured with a 15 s cut-off time (Zelena et al. 2009b). Right at the end of negative geotaxis test, the pups were decapitated, and trunk blood and hypophysis were collected.

Hormone measurements

Blood was collected on ice-cold Eppendorf tubes and centrifuged at 3000 rpm for 30 min at −4 °C. Serum was stored at −20 °C till hormone measurements. From serum samples, ACTH and corticosterone concentrations were measured by specific radioimmunoassay (RIA) without previous extraction. Both antibodies were developed in our Institute as described elsewhere (Zelena et al. 1999, 2003a, b). The detection limits were 4 fmol/ml for ACTH and 2.7 pmol/ml for corticosterone. The intraassay coefficients of variation were 4.7 for ACTH and 12.3 for corticosterone.

In the case of mixed litters (Exp. 1 and 3), where the genotype of individuals was not obvious, we also collected the hypophysis of the pups to determine AVP content. Pituitary samples were stored in 100 µl 0.1 N HCl at −20 °C. The preparation of the samples was as follows: they were placed in a boiling water bath for 5 min, homogenized by ultrasound, and centrifuged, and AVP content was measured from the 100-fold diluted supernatant using specific RIA. The rabbit antibodies were donated by Dr. M. Vecsernyés (University of Debrecen, Hungary). The limit of detection was 1 pg AVP/assay tube. ^{125}I-labeled tracers were produced by the chloramine-T method. Bound and free fractions were separated by charcoal.

All the samples from a particular experiment were assayed in the same RIA.

Statistical analyses

Data were analyzed by one-way (Exp. 1 factor: genotype; Exp. 2 factor: treatment) or two-way ANOVA (Exp. 3 factors: genotype and treatment) using the Statistica 11.0 program of StatSoft, Inc., Tulsa, OK, USA. Post-hoc comparison of the data from different experimental groups was performed by the Newman–Keuls test. Data are presented as mean ± SEM. The level of statistical significance was taken as $p < 0.05$.

Results

Genotypes

The AVP content of the whole pituitary was under the detection limit in AVP-deficient (d*i*/d*i*) animals (Table 1). Statistically the effect of genotype was significant [$F_{(2,65)} = 130.1$; $p < 0.01$].

In case of the body weight, there was a highly significant influence of the pup's genotype [$F_{(2,72)} = 7.6$; $p < 0.01$] (Table 1). More specifically, the d*i*/d*i* animals were smaller that their d*i*/+ littermates or age-matched +/+ pups.

Both the vocalization duration [$F_{(2,72)} = 4.2$; $p < 0.05$] and frequency [$F_{(2,72)} = 5.2$; $p < 0.01$] showed significant genotype effect (Fig. 1a, b). It means that the d*i*/d*i* rats emitted significantly less USV than their d*i*/+ littermates or +/+ animals. Representative USV samples showed similar picture (Fig. 1e–g).

Regarding the HPA axis hormones, there was a significant genotype effect in case of ACTH levels [$F_{(2,72)} = 3.4$; $p < 0.05$] (Fig. 1c). More specifically, the d*i*/d*i* animals represented lower ACTH levels than their littermates.

Table 1 Pituitary AVP content and body weight differences between the 3 genotypes

	+/+	d*i*/+	d*i*/d*i*
AVP (ng/pituitary)	86.6 ± 4.9	57.7 ± 3.7**	1.8 ± 0.3**,##
Body weight (g)			
Exp. 1	17.41 ± 0.2	16.35 ± 0.5	15.20 ± 0.4**,#
Exp. 2	16.04 ± 0.3		
Exp. 3		16.08 ± 0.5	14.12 ± 0.25##

** $p < 0.01$ vs +/+, # $p < 0.05$, ## $p < 0.01$ vs d*i*/+

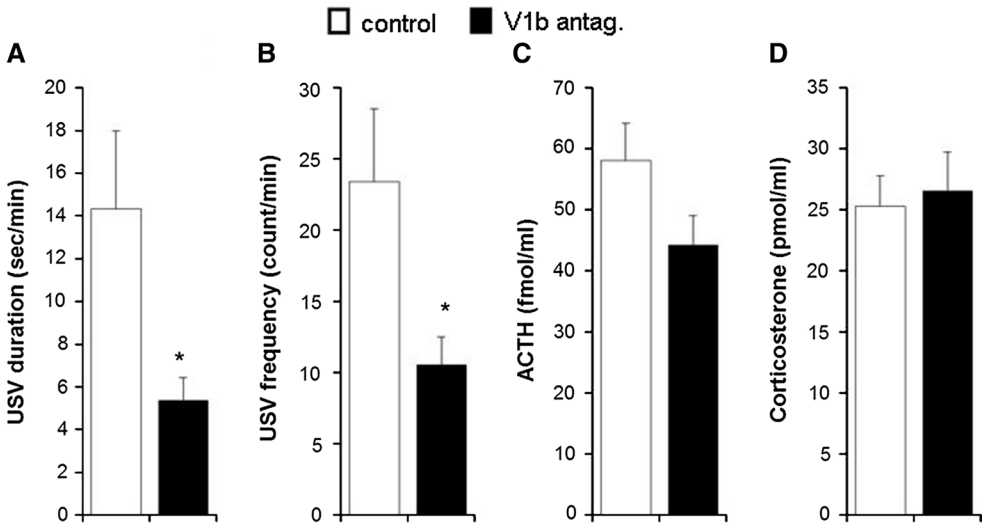

Fig. 2 The effect of SSR149415 (10 mg/kg ip 30 min prior to test) in 7- to 8-day-old +/+ Brattleboro pups. *$p < 0.05$ vs. control, vehicle treated animals

Although the levels of corticosterone also showed significant genotype effect [$F_{(2,72)} = 83.1$; $p < 0.01$], in this case, d*i*/d*i* animals represented an elevation and not a reduction compared to both other genotypes (Fig. 1d).

There was a negative correlation between serum corticosterone levels and USV duration [$F_{(1,73)} = 7.42$; $p < 0.01$; beta = −0.30], as well as between corticosterone and USV frequency [$F_{(1,73)} = 8.71$; $p < 0.01$; beta = −0.33].

V1bR antagonist

SSR149415 treatment reduced the USV duration [$F_{(1,43)} = 5.45$; $p < 0.05$] and frequency [$F_{(1,43)} = 5.35$; $p < 0.05$] (Fig. 2a, b).

The accompanied ACTH [$F_{(1,43)} = 2.16$; $p = 0.15$] and corticosterone [$F_{(1,43)} = 0.62$; $p = 0.43$] levels were not effected by the treatment (Fig. 2c, d).

There was a negative correlation between corticosterone levels and USV frequency [$F_{(1,42)} = 4.16$; $p < 0.05$; beta = −0.30], while its interaction with USV duration was only marginally significant [$F_{(1,42)} = 3.82$; $p = 0.05$; beta = −0.29].

CDP

Both the USV duration [$F_{(1,68)} = 13.52$; $p < 0.01$] and frequency [$F_{(1,69)} = 14.37$; $p < 0.01$] were significantly smaller in d*i*/d*i* compared to d*i*/+ pups (Fig. 3a, b). CDP significantly reduced USV duration [$F_{(1,68)} = 6.00$; $p < 0.05$] and frequency [$F_{(1,69)} = 6.00$; $p < 0.05$]. However, we can establish that the effect of CDP was detectable only in d*i*/+, but not in d*i*/d*i* animals, as there was a significant interaction between the genotype and treatment both in case of USV duration [$F_{(1,68)} = 4.32$; $p < 0.05$] and frequency [$F_{(1,69)} = 4.00$; $p < 0.05$].

The serum ACTH levels were significantly lower in d*i*/d*i* rats compared to their littermates [$F_{(1,69)} = 37.24$; $p < 0.01$] without any effect of the treatment [$F_{(1,69)} = 2.61$; $p = 0.11$] or without any significant interaction [$F_{(1,69)} = 1.89$; $p = 0.17$] (Fig. 3c). On the contrary, the corticosterone levels were higher in d*i*/d*i* pups [$F_{(1,66)} = 9.58$; $p < 0.01$] without treatment effect [$F_{(1,66)} = 0.06$; $p = 0.80$] or interaction [$F_{(1,66)} = 1.02$; $p = 0.32$] (Fig. 3d).

There was a significant positive correlation between USV duration and ACTH levels [$F_{(1,70)} = 4.80$; $p < 0.05$; beta = +0.25], USV frequency and ACTH levels [$F_{(1,70)} = 5.16$; $p < 0.05$; beta = +0.26], while corticosterone levels revealed negative correlations with these parameters [USV duration ($F_{(1,67)} = 4.63$; $p < 0.05$; beta = −0.25), USV frequency ($F_{(1,67)} = 5.04$; $p < 0.05$; beta = −0.26)].

There was no difference between the groups in the latency of negative geotaxis suggesting an absence of sedation (data not shown).

Discussion

In the present experimental series, we were able to detect the supposed USV-reducing effect of CDP treatment (Gardner 1985; Hodgson et al. 2008; Olivier et al. 1998), which was similar to the anxiolytic effect of the genetic (Brattleboro rat) and pharmacological (SSR149415) AVP deficiency. AVP deficiency prevented the effect of CDP on USV. Despite our expectation, the plasma stress-hormone levels did not go parallel with the behavior. In contrast, serum corticosterone levels showed negative correlation with the measured USV parameters.

Our results are in line with previous observations. Winslow and Insel (1993) found that subcutaneous

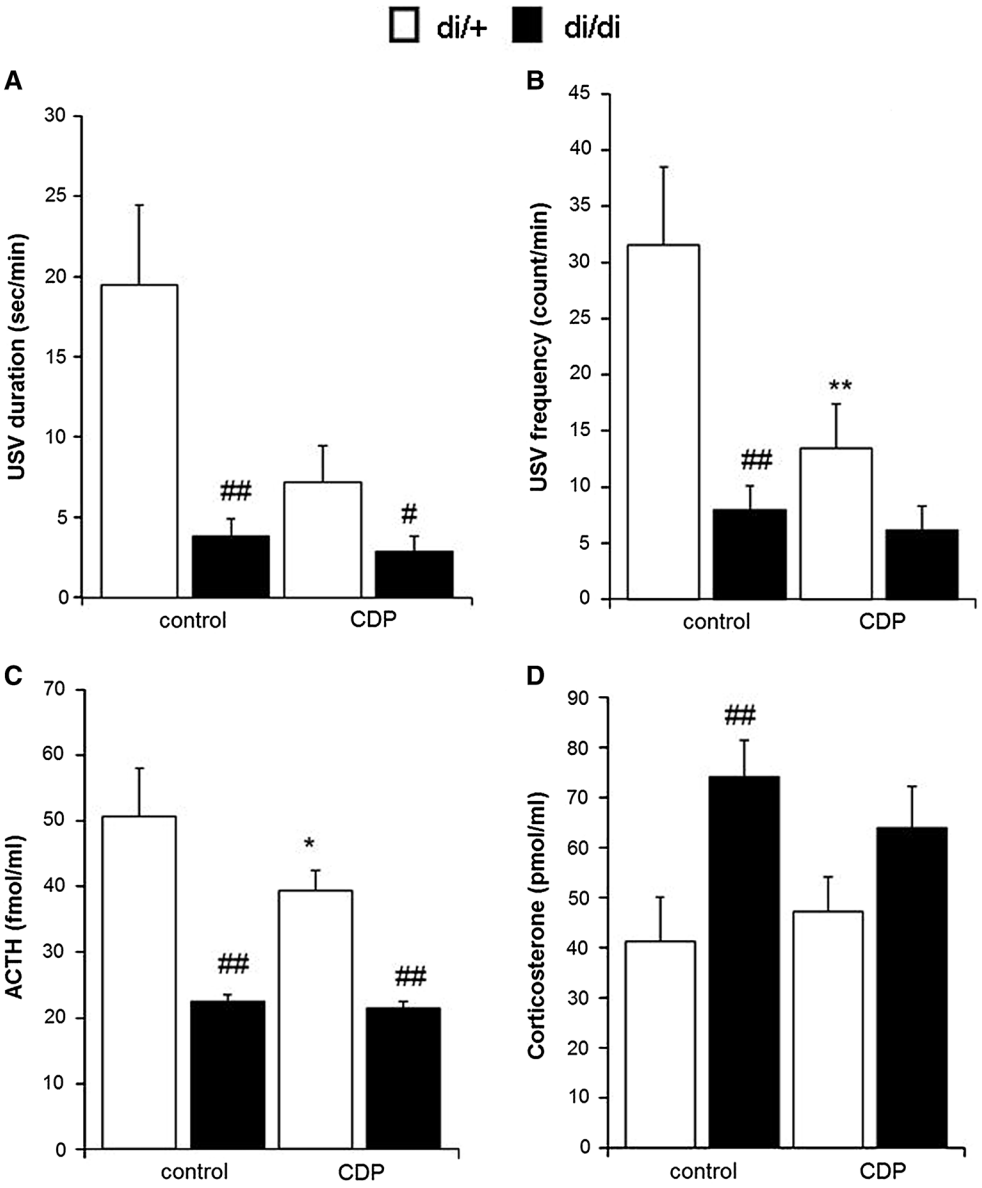

Fig. 3 The effect of chlordiazepoxide (CDP) administration (10 mg/kg ip 30 min prior test) in 7- to 8-day-old heterozygous (d*i*/+) and homozygous AVP-deficient (d*i*/d*i*) rats. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control, saline treated rats; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. d*i*/+

administration of AVP increased USV during a 2 min test. On the contrary, central administration of AVP was reported to decrease callings. Because this decrease was accompanied by reduced locomotion and disturbed negative geotaxis as general signs of sedation, the central AVP effect on USV might be due to a sedative and not to an anxiolytic effect. Our results in Brattleboro rat support also the observations of Lin et al. (2013). In their hands, the intercall intervals were significantly longer in d*i*/d*i* pups than in wild-types; however, during the 2 min recording period, the frequency of calls was not significantly different between the genotypes.

Take into consideration, the developmental role of AVP (Petracca et al. 1986; Zelena et al. 2009b) in Brattleboro pups secondary changes might be also responsible for the observed effects. Therefore, pharmacological validation with short, acute treatment was necessary. In our hand, 10 mg/kg SSR149415 was effective, despite a previous study reporting only a tendency during 5-min test period (Iijima and Chaki 2005). Possible explanation of this discrepancy can be related to some chemical aspect (the source and solution of the compound was different), but it is also possible that longer observation was necessary for the development of the drug effect. Indeed, in a subsequent study, SSR149415 effectively reduced the USV duration and frequency during a 10 min test both in rats and guinea pigs, although only in the 30 mg/kg dose (Hodgson et al. 2007). Even the high dose (30 mg/kg) was unable to induce sedative side effect measured by righting reflex and negative geotaxis (Hodgson et al. 2007), suggesting a direct anxiolytic contribution. The effect of SSR149415 was thought to go through the V1b receptors in the pituitary

thereby through HPA axis regulation (Aguilera et al. 2008; Serradeil-Le Gal et al. 2003), but our present results (no correlation between ACTH and behavior) suggest that in the effect of SSR149415, other brain areas [or peripheral receptors (Gallo-Payet and Guillon 1998)] could be also involved. Previous observations also concluded that in the effect of SSR149415, extrahypothalamic brain sites could be involved (Ramos Ade et al. 2014). In another model, in the V1bR knockout mice, the standard USV measures were not different between the genotypes, but the lack of the V1bR prevented the expected increase in vocalizations during the second separation from the mother and siblings (maternal potentiation USV paradigm) (Scattoni et al. 2008).

We have to be aware that V1aRs might be also related to anxiety (Zelena 2012). As V1aRs cannot be found at the pituitary and are not directly linked to HPA axis regulation, they may provide the suggested extrahypothalamic link between AVP and anxiety most probably through the septum (Bleickardt et al. 2009; Liebsch et al. 1996). Indeed, high dose of a V1aR antagonist (given ip 30 min prior testing) was found to reduce USV in rat pups (measured for 10 min) (Bleickardt et al. 2009).

Our results showed that the effect of GABA blockade by CDP (Macdonald and Barker 1978) was abolished in AVP-deficient rats. Although theoretically it would be possible that d*i*/d*i* pups vocalize at a very low level that is impossible to diminish further, using a $GABA_B$ agonist (baclofen) in 2 mg/kg dose, we confirmed further decrease in USV (data not shown). Thus, there is an interaction between AVP and the GABAergic system on anxiety regulation already during the postnatal period. Based on adult data, GABA is known to inhibit the electrical and secretory activities of AVPergic neurons located in the supraoptic and paraventricular nuclei following osmotic, cardiovascular, or suckling stimuli (Fenelon and Herbison 1995). However, the osmoregulation within the supraoptic nucleus might involve taurine as main inhibitory transmitter rather than GABA (Engelmann et al. 2001; Hussy et al. 1997). In conjunction with anxiety, SSR149495 was able to diminish an anxiogenic benzodiazepine inverse agonist (FG7142)-induced acetylcholine elevation in hippocampus (Claustre et al. 2006). Moreover, temazepam, a representative classical benzodiazepine, enhanced the AVP secretion within the hypothalamus (Welt et al. 2006). This intranuclear AVP elevation may contribute to the HPA axis calming effect of temazepam during stress (Zelena et al. 2009a). On the contrary, midazolam, a short acting benzodiazepine, reduces anesthesia-induced AVP elevation in human (Sjovall et al. 1983). Additional to its central nervous system effect, midazolam inhibits AVP-induced accumulation in heat shock protein 27 in vascular smooth muscle (Tanabe et al. 2001). In line with this peripheral effect CDP was shown to decrease basal plasma AVP levels (Wible et al. 1985) as well as footshock-induced elevations (Yagi and Onaka 1996). Alprazolam, another benzodiazepine binding specifically to GABAA receptors, suppresses the AVP-induced ACTH and cortisol elevation in human (Torpy et al. 1994; Vicennati et al. 2004).

Enhanced HPA axis activity is accompanied by elevated anxiety level both in rodents and in humans (Abelson et al. 2007; Landgraf et al. 1999). A brief period of neonatal isolation in a temperature-controlled environment without the presence of familiar sensory cues results in a significant increase in plasma corticosterone levels (McCormick et al. 1998; Zelena et al. 2008). On the other hand, most of the previous studies have demonstrated that classical benzodiazepines such as CDP decrease the HPA axis activity in stressful contexts (Arvat et al. 2002; Kalman et al. 1997; Pomara et al. 2005). Similarly, the genetic [Brattleboro rat (Buckingham and Leach 1980; McCann et al. 1966)] as well the pharmacological diminution of the AVP activity was shown to reduce the HPA axis activity not only in adult rats but also during the postnatal period (Torpy et al. 1994; Zelena et al. 2009b). Moreover, during the postnatal period, AVP seems to be the main regulator of the HPA axis [for details about AVPergic HPA axis regulation see (Makara et al. 2012)]. Our present results confirm a reduction in ACTH secretion in AVP-deficient animals, which went parallel with reduced USV (whereas only in one out of three experiments).

Interestingly, the corticosterone plasma levels were even higher, and there was a negative correlation between USV and serum glucocorticoid level in all three experiments. The inverse relationship between vocalization and cortisol could be predicted by the coping model suggested by Levine and Wiener (1988). Accordingly, vocalization is a coping response which acts to reduce the apparent stressfulness of the experience for the infant and thus to reduce the concurrent adrenocortical response in the setting of the adjacent mother. In line with this assumption the infant Rhesus Monkeys' vocalization response was higher when they were separated physically (remained in visual, auditory, and olfactory contact = milder stressor exposure) than if the infant was separated totally from their mother (Bayart et al. 1990). On the contrary, its adrenocortical response to isolation was the opposite showing the highest response in complete isolation. Nevertheless, it is quite obvious that this corticosterone rise is not driven by ACTH [for details about ACTH-corticosterone dissociation see (Bornstein et al. 2008)].

As a summary, using a maternal separation-induced USV paradigm, we confirmed our hypothesis that AVP has an anxiogenic role already during early postnatal ages. Because anxiolytic effect did not go parallel with diminished HPA axis activation, other than V1bRs could also

be targeted in the therapy. Moreover, AVP antagonists and benzodiazepines might diminish each other's effect, as AVP deficiency interacted with CDP. An alternative explanation of our results is that enhanced USV represents an active coping style resulting in reduction of glucocorticoid levels.

Acknowledgments Experiments were carried out in accordance with the European Communities 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, Budapest, Hungary. The manuscript does not contain clinical studies or patient data. All persons gave their informed consent prior to their inclusion in the study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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