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Low-dose antipsychotics ameliorate maternal separation-induced ultrasound vocalization deficits in the vasopressin-deficient Brattleboro rat pups

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ABSTRACT

Beyond its classical function in salt-water homeostasis, vasopressin plays an important role in higher-order brain functions, including social behaviour. Deficits in social communication are core feature of many neurological and psychiatric disorders, including autism spectrum disorder (ASD), and can be modelled in rodents through maternal separation-induced ultrasonic vocalizations (MS-USV, 30–50 kHz). Consistent with its role in social interaction, vasopressin-deficient Brattleboro rat pups emit fewer MS-USVs. We hypothesized that antipsychotics, approved for irritability and related behavioural manifestations in ASD, could normalize this deficit.

We studied 7- to 8-day-old vasopressin-deficient Brattleboro pups and compared them with their heterozygous littermates. Each pup was placed in a 2 L glass beaker for 10 min while MS-USV was recorded using a bat detector. We administered haloperidol, clozapine, olanzapine, risperidone, aripiprazole or vehicle and the genotype was determined retrospectively.

Vasopressin-deficient pups vocalized significantly less than their counterparts. Low, non-sedative doses of various antipsychotics (except haloperidol) increased MS-USV in vasopressin-deficient pups to levels comparable to controls. However, higher doses, which induced sedation (measured by increased righting reflex latency or negative geotaxis latency) or stress (indicated by elevated adrenocorticotropin and corticosterone levels), reduced MS-USV in control pups as well.

These findings indicate that reduced MS-USV in vasopressin-deficient Brattleboro pups provides a useful model for studying impairments in early-life social communication, which may also be highly relevant in the context of ASD. The responsiveness to low-dose pharmacological intervention further supports the model's relevance for investigating mechanisms and potential therapeutic strategies targeting social behavioural alterations.

1. Introduction

Social communication is fundamental to adaptive functioning, enabling individuals to interpret social cues, express intentions, and engage in reciprocal interactions. Disruptions in social communication are a core or prominent feature across multiple neurodevelopmental and psychiatric disorders (Fett et al., 2011; Hoza, 2007; Kupferberg et al., 2016; Samame et al., 2012), including autism spectrum disorder (ASD) (Mandy et al., 2017). In more severe forms of ASD, the patients are often unable to learn to speak and care for themselves, placing a heavy burden on education, health, and social care. Growing evidence suggests that atypical early vocalizations, such as altered crying, may represent one of the earliest detectable features of ASD and serve as an early biomarker in at least a subset of affected individuals (Esposito et al., 2017). Across diagnoses, social communication deficits are associated with poorer long-term psychosocial outcomes, increased caregiver stress, and

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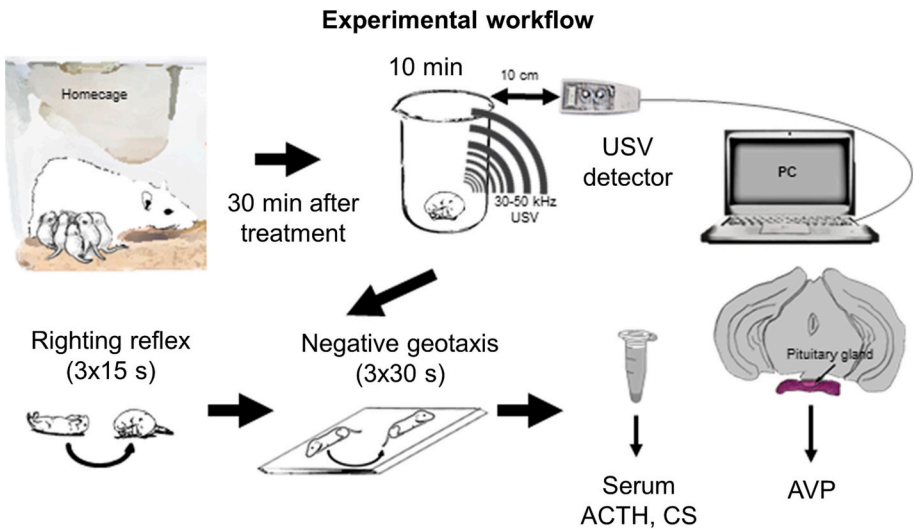

Fig. 1. Experimental workflow. Abbreviations: ACTH: adrenocorticotropic hormone; AVP: arginine vasopressin; CS: corticosterone; USV: ultrasonic vocalization.

reduced quality of life (Fett et al., 2011; Green et al., 2015; Hayes and Watson, 2013; Kupferberg et al., 2016). Accordingly, understanding the neurobiological mechanisms underlying social communication is critical for identifying transdiagnostic therapeutic targets and improving functional outcomes across a range of neurological and psychiatric conditions.

Arginine-vasopressin (AVP), beside its well-known role in salt-water homeostasis (Decaux et al., 2008), is a key player in the regulation of complex social behaviours in mammals (e.g. regulation of aggression, pair bonding and parental behaviour, memory, social recognition) (Caldwell et al., 2008). Indeed, AVP is present early in the brain, and has various mitogenic, metabolic and physiological actions (Boer, 1985; Yoshimura et al., 2021). Thus, one might expect AVP to be of importance for normal brain development (Boer, 1985) and was implicated in several aspects of ASD symptomatology (Clarke et al., 2024; Laszlo et al., 2023). Moreover, in children with ASD (mostly males) intranasal AVP was showed to be an effective treatment (Parker et al., 2019). Other authors also supported the notion that AVP plays sex-specific role in ASD (Carter, 2007).

Brattleboro rats are naturally occurring models of genetic AVP deficiency. They have a spontaneous mutation in the neurophysin region of AVP precursor gene, which leads to inappropriate AVP processing and lack of functional AVP in the brain (Guldenaar and Pickering, 1988; Krisch et al., 1986; Sausville et al., 1985; Schmale et al., 1984; Valtin and Schroeder, 1964). They have a number of social and cognitive abnormalities, like impaired social recognition (Engelmann and Landgraf, 1994). The memory-, attention-, and communication deficit of adult Brattleboro rats was confirmed by another group (Cilia et al., 2010) and in our colony (Demeter et al., 2016; Fodor et al., 2016) as well. These behavioural abnormalities of AVP-deficient rats (especially the prepulse inhibition deficit) can be treated by antipsychotic but not psychotropic drugs of different therapeutic families (e.g. imipramine, diazepam, valproic acid) (Cilia et al., 2010; Feifel et al., 2004; Feifel et al., 2009; Feifel and Priebe, 2001; Torok et al., 2021a).

However, because communication difficulties can emerge early in life, especially in ASD, research should extend beyond adult phenotypes to include early postnatal developmental stages. During maternal separation rat pups emit ultrasonic vocalizations (MS-USV) with the aim to restore tactile, olfactory and thermal conditions associated with the nest. Therefore studying MS-USV could be a good measure of early social communication (Sampino et al., 2014; Seffer et al., 2012). Indeed, it was repeatedly demonstrated that various mouse models of ASD display severe deficits in MS-USV production in line with their affiliative communicative function (Laszlo et al., 2024; Wohr, 2014; Wohr and Scattoni, 2013; Wöhr and Schwarting, 2010). Unfortunately, ASD can be associated with a variety of severe comorbid disorders, such as major depression, anxiety, and even diminished or unusual emotional expression. AVP level can positively correlate with the manifestation of anxiety symptoms in adulthood (Keck, 2006). As MS-USV is often used as an anxiety model (Groenink et al., 2008), the previously found MS-USV-deficit in the Brattleboro pups was interpreted as reduced anxiety (Varga et al., 2015), in accordance with a similar, less anxious phenotype of the adult Brattleboro rats (Mlynarik et al., 2007). However, MS-USV was independent from the stress-axis regulation (Torok et al., 2021b). Here we hypothesized that lower MS-USV emitted by AVP-deficient Brattleboro pups (Varga et al., 2015) provides a useful model for studying impairments in early-life social communication, with particular relevance to ASD, where early deficits in social interaction and communication are among the core diagnostic features.

In human patients with ASD, antipsychotic treatment is approved and widely used for the improvement of disruptive behavioural symptoms like irritability, psychosis, and aggression (Bartram et al., 2019; Posey et al., 2008; Shafiq and Pringsheim, 2018). However, communication impairments themselves can contribute to irritability and aggression in ASD (Neuhaus et al., 2022; Tordjman, 2022). When individuals experience difficulties in expressing themselves, frustration may accumulate and subsequently manifest as irritability or aggressive behaviour (Fipp-Rosenfield et al., 2024; Tordjman, 2022). Moreover, several studies have also reported beneficial effects of antipsychotics on social and communicative functioning (Campbell et al., 1978; Nagaraj et al., 2006; Shea et al., 2004).

The first generation (typical) antipsychotics (FGA) (e.g. *haloperidol*) are antagonists for dopamine D2 receptors with high affinity. Second (SGA) (e.g. *clozapine, olanzapine, risperidone*) and third generation antipsychotics (TGA) (*aripiprazole*) (SGA and TGA are together atypical) have been approved also for the treatment of other psychiatric disorders such as ASD (de Araujo et al., 2012). Risperidone is the only atypical neuroleptic that has obtained FDA approval for use in children and adolescents with ASD (olanzapine and aripiprazole need more research before recommendations can be made for their use) (Canitano and Scandurra, 2011).

We used haloperidol, clozapine, olanzapine, risperidone and aripiprazole to test our hypothesis. As higher doses of the antipsychotics might have sedative effects, which may induce anxiolytic-like changes (e.g. reduced USV) (Hodgson et al., 2008), we used rather small doses (Kapur et al., 2003) and tested also some behavioural markers (righting reflex and negative geotaxis) of sedation (Torok et al., 2021b; Zelena et al., 2009). Although these doses were smaller than clinically relevant

Table 1
Main effects of the two-way ANOVA analysis.

	Weight	RRL	Negative geotaxis	USV frequency	USV duration	ACTH	CS
Haloperidol (0.1 and 1 mg/kg)							
F(1,88) genotype	82.6 p<0.01	2,7 p=0.1	0.3 p=0.6	8.3 p<0.01	15.4 p<0.01	68.9 p<0.01	30.2 p<0.01
F(2,88) treatment	0.34 p=0.7	1,0 p=0.37	1.1 p=0.34	1.1 p=0.33	0.9 p=0.4	35.3 p<0.01	4.6 p=0.01
F(2,88) interaction	1.5 p=0.23	0,7 p=0.49	1.2 p=0.32	1.86 p=0.16	1.8 p=0.16	1.88 p=0.15	0.7 p=0.47
Clozapine (1 and 10 mg/kg)							
F(1,69) genotype	9.9 p<0.01	2.7 p=0.1	0.8 p=0.36	8.7 p<0.01	8.1 p<0.01	40.72 p<0.01	13.8 p<0.01
F(2,69) treatment	0.28 p=0.75	37.15 p<0.01	16.9 p<0.01	15.5 p<0.01	10.7 p<0.01	6.9 p<0.01	8.8 p<0.01
F(2,69) interaction	1.65 p=0.2	0.15 p=0.85	0.16 p=0.84	2.7 p=0.073	2.4 p=0.09	5.0 p<0.01	3.0 p=0.05
Olanzapine (0.3 and 3 mg/kg)							
F(1,86) genotype	96.1 p<0.01	10.0 p<0.01	0.004 p=0.95	12.96 p<0.01	12.57 p<0.01	88.6 p<0.01	22.8 p<0.01
F(2,86) treatment	2.65 p=0.07	147.5 p<0.01	166.2 p<0.01	34.2 p<0.01	30.6 p<0.01	41.6 p<0.01	12.4 p<0.01
F(2,86) interaction	0.06 p=0.9	2.2 p=0.12	2.3 p=0.11	3.94 p<0.05	4.1 p<0.05	20.3 p<0.01	0.6 p=0.55
Risperidone (0.25 and 1 mg/kg)							
F(1,91) genotype	30.5 p<0.01	6.41 p=0.01	0.29 p=0.59	884.5 p<0.01	7.37 p<0.01	10.54 p<0.01	331.9 p<0.01
F(2,91) treatment	2.3 p=0.1	11.74 p<0.01	16.7 p<0.01	0.94 p=0.39	15.2 p<0.01	8.9 p<0.01	149.6 p<0.01
F(2,91) interaction	0.6 p=0.55	1.83 p=0.17	3.25 p<0.05	0.81 p=0.44	3.4 p<0.05	2.24 p=1.11	58.2 p<0.01
Risperidone (0.05 and 0.1 mg/kg)							
F(1,90) genotype	46.8 p<0.01	15.7 p<0.01	0.77 p=0.38	12.13 p<0.01	19.4 p<0.01	61.9 p<0.01	71.4 p<0.01
F(2,90) treatment	0.46 p=0.62	1.45 p=0.24	1.45 p=0.24	1.14 p=0.32	2.71 p=0.07	12.2 p<0.01	30.9 p<0.01
F(2,90) interaction	1 p=0.37	0.46 p=0.64	0.99 p=0.37	2.27 p=0.1	1.05 p=0.35	5.71 p<0.01	3.95 p<0.01
Aripiprazole (0.5 and 5 mg/kg)							
F(1,134) genotype	68.8 p<0.01	19.3 p<0.01	1.6 p=0.2	11.2 p<0.01	18.9 p<0.01	60.2 p<0.01	97.2 p<0.01
F(2,134) treatment	0.21 p=0.81	4.84 p<0.01	0.29 p=0.75	5.76 p<0.01	4.47 p=0.01	18.9 p<0.01	3.8 p<0.05
F(2,134) interaction	2.93 p=0.056	8.7 p<0.01	4.85 p<0.01	0.46 p=0.63	0.76 p=0.46	2.02 p=0.14	1.72 p=0.18
Aripiprazole (50 mg/kg)							
F(1,56) genotype	7.2 p<0.01	9.8 p<0.01	9.35 p<0.01	5.28 p<0.05	5.09 p<0.05	148.6 p<0.01	28.7 p<0.01
F(1,56) treatment	0.7 p=0.42	2.24 p=0.14	6.2 p=0.01	0.002 p=0.96	0.07 p=0.79	72.6 p<0.01	5.45 p<0.05
F(1,56) interaction	0.09 p=0.76	2.7 p=0.1	5.1 p<0.05	1.66 p=0.2	1.16 p=0.028	32.2 p<0.01	0.09 p=0.76

Significant differences are marked as red, while trends as blue.

concentrations, the blood-brain-barrier (BBB) of the pups is functionally immature concomitantly with structural immaturity (Gomez-Gonzalez and Escobar, 2009). Therefore, with these smaller doses we may reach similarly effective brain concentrations than with higher doses in adults. MS-USV in pups is induced by a stressor (MS + cold) and AVP plays an important role in the regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis (also called stress-axis). Therefore it seemed reasonable to check the stress hormone (adrenocorticotrophic hormone: ACTH and corticosterone) release, too, as one of the main background mechanism of adaptation (Aguilera, 1994; Selye, 1975). Dysregulation of the HPA system may contribute to development of ASD as well (Brosnan et al., 2009; Kidd et al., 2012; Tordjman et al., 2014).

2. Materials and methods

2.1. 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 7–8-day-old Brattleboro pups from the same litters with mating homozygous AVP deficient (di/di) fathers and heterozygous (di/+) mother rats; in this way di/+ and di/di rats were born (Bohus and de Wied, 1998; Zelena et al., 2003b). The litter size was not controlled, and sex was not taken into consideration. The genotype of the pups was assessed after the experiment upon the AVP content of the pituitary by radioimmunoassay. 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.

Because MS-USVs were measured sequentially, animals were tested one at a time. The testing sequence was as follows (Fig.1): Pups were injected subcutaneously, marked, and returned to the dam. Thirty min later, they were individually tested for MS-USVs in a 2 L jar for 10 min. Subsequent pups were tested sequentially at 12 min intervals. After MS-USV recording, righting reflex and negative geotaxis tests were performed, and pups were sacrificed within 5 min of completing behavioural testing. This protocol was applied consistently to each animal.

2.2. Drugs

Pups were injected subcutaneously with 1 μL/g antipsychotic drug or vehicle 30 min before the MS-USV test. Different doses were used for *haloperidol* (0.1 mg/kg, 1 mg/kg; Sigma Chemical Co., St. Louis, MO), *clozapine* (1 mg/kg, 10 mg/kg; Sigma Chemical Co., St. Louis, MO), *olanzapine* (0.3 mg/kg, 3 mg/kg; Gedeon Richter Plc., Budapest, Hungary), *risperidone* (0.25 mg/kg, 1 mg/kg, 0.05 mg/kg, 0.1 mg/kg; Sigma Chemical Co., St. Louis, MO) and *aripiprazole* (0.5 mg/kg, 5 mg/kg, 50 mg/kg; Gedeon Richter Plc., Budapest, Hungary). Haloperidol, clozapine, olanzapine and risperidone were dissolved in 50 μL of 1 N HCl per mL of saline solution, while aripiprazole was dissolved in 5% acetic acid-saline solution and titrated with 3 N NaOH to pH 6–7. Control animals got vehicle (HCl or acetic acid). The sample size was 9–28/group (for details see Supplementary Table 1.).

2.3. Maternal separation-induced ultrasonic vocalization: MS-USV

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 behavioural test. 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. Pups received an injection of the drug or vehicle (1 μL/g) one after the other in every 12 min, they were marked and returned to the dam and littermates (Torok et al., 2021b; Varga et al., 2015). Thirty min after the administration one pup was brought to a soundproof room and placed in a 2 L glass beaker without bedding and heating. MS-USV was measured for 10 min. Individual calls during this period were detected using an ultrasound-sensitive frequency division detector (CDB205, CIEL Electronique, Nice, France) fixed on a holder 25 cm above the bottom of the glass beaker connected to a computer. The distance between the USV detector and the beaker was 10 cm. Vocalizations were recorded using a free Audacity 2.0.5. software and stored on a personal computer. In previous studies, the large portions of ultrasonic vocalizations emitted by 8-day-old rats were found from 30 kHz to 50 kHz (Allin and Banks, 1971; Blumberg et al., 2000). Thus, signals were filtered, and the power spectrum was analysed ranging from 30 kHz 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.

Although detailed multi-parametric analyses of USVs are informative for studies focusing on social communication and vocal repertoire structure (Nakamura et al., 2021; Takahashi et al., 2016), the primary aim of the present study was pharmacological screening rather than fine-grained phenotypic characterization. Therefore, in line with long-

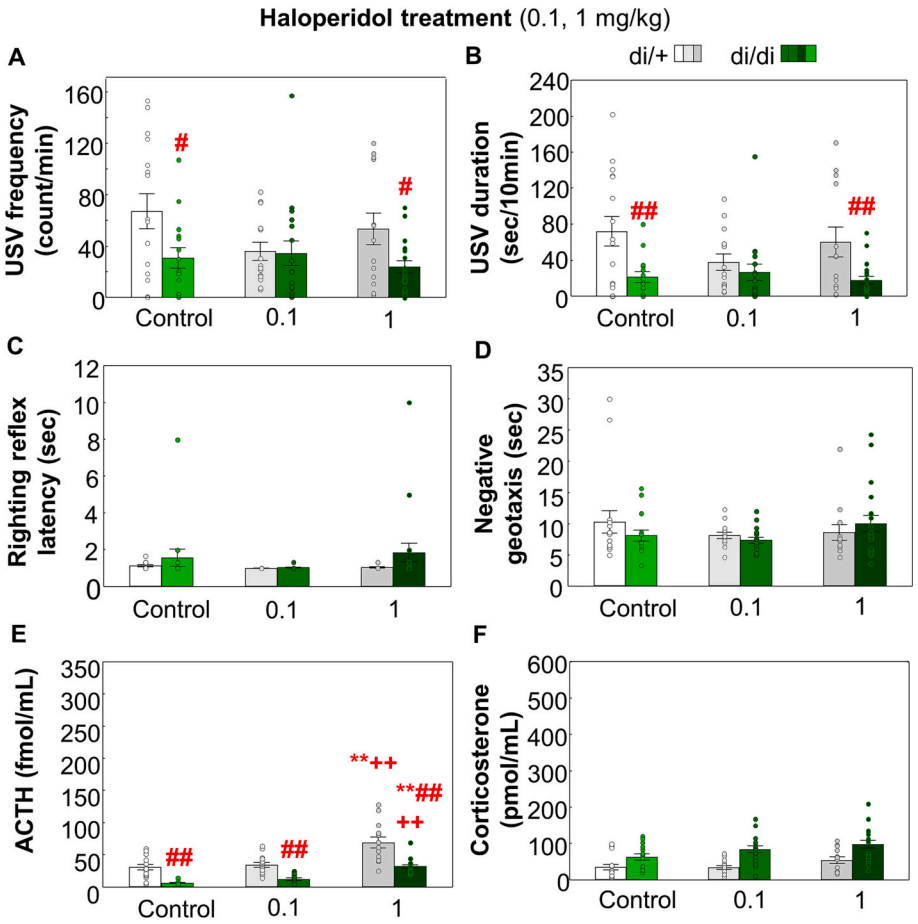

Fig. 2. Haloperidol treatment (0.1, 1 mg/kg). A) USV frequency (count/min) 30 min after treatment; B) USV duration (sec/10 min) 30 min after treatment; C) Righting reflex latency (sec) right after MS USV measurement; D) Negative geotaxis (sec) right after MS USV measurement; E) ACTH serum concentration (fmol/mL) after MS USV measurement; F) Corticosterone serum concentration (pmol/mL) after MS USV measurement. #p < 0.05, ##p < 0.01 significant difference from respective di/+ animals; **p < 0.01 significant difference from vehicle treated rats; ++*p* < 0.01 significant difference from the other treated group.

standing practice in pharmacological and stress-related USV studies (Hodgson et al., 2008; Hofer, 1996; Venkatraman et al., 2024; Yin et al., 2016), we employed a simple, robust, and time-efficient analytical approach based on a limited number of well-defined parameters (namely USV frequency calculated as the total number /10 min and total duration of calls per session) that can be reliably reproduced across experimental cohorts.

2.4. Behaviour

Righting reflex latency (RRL) test was used for investigating sedative effect of the drugs (Zelena et al., 2009). Offspring were placed into an unusual supine posture on a smooth, flat surface and the latency to return the normal upright position with all four feet on the table was measured manually. Each animal was allowed a maximum of 30 s to return to prone position.

Negative geotaxis test investigated impaired motor coordination. Offspring were placed on a 45° inclined foam-rubber board with their nose pointing downward and its latency to turn 180° to an upright position was recorded in seconds. Each animal was allowed a maximum of 60 s on the apparatus to complete the task. If the rat fell off the plane, the rat was considered to have failed the task and a maximum score of 60 s was assigned.

Both tests were repeated three times at the termination of the measurement of the USV and the mean of the three measurements was taken as a typical parameter.

2.5. Hormone measurements

Although behavioural testing can activate the HPA axis, this activation is slower in pups than in adults due to the stress hyporesponsive period (Sapolsky and Meaney, 1986; Schmidt et al., 2004). Therefore, our standardized behavioural protocol is unlikely to have significantly affected the measured outcomes.

Pups were immediately decapitated after behavioural measurements and 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 the hormone assay. From serum samples ACTH and corticosterone concentrations were measured by specific radioimmunoassay (RIA) without previous extraction. Both antibodies were developed at the Institute of Experimental Medicine, Budapest, Hungary as described elsewhere (Zelena et al., 1999; Zelena et al., 2003a). The intra-assay coefficients of variations were 4.7% and 7.5%, respectively for the two hormones. All samples from one experiment was measured in one assay.

The hypophysis of the pups was also collected to determine the AVP content thereby the genotype. Pituitary samples were stored in 100 μL of 0.1 N HCl at −20 °C. The preparation of the samples were as following: they were placed in a boiling water bath for 5 min, and then homogenized by ultrasound, centrifuged, and AVP content was measured from the 100-fold diluted supernatant using specific RIA as described earlier (Zelena et al., 2009). The rabbit antibodies were donated by Dr. M. Vecsernyés (Szent-Györgyi Medical University, Szeged, Hungary).

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

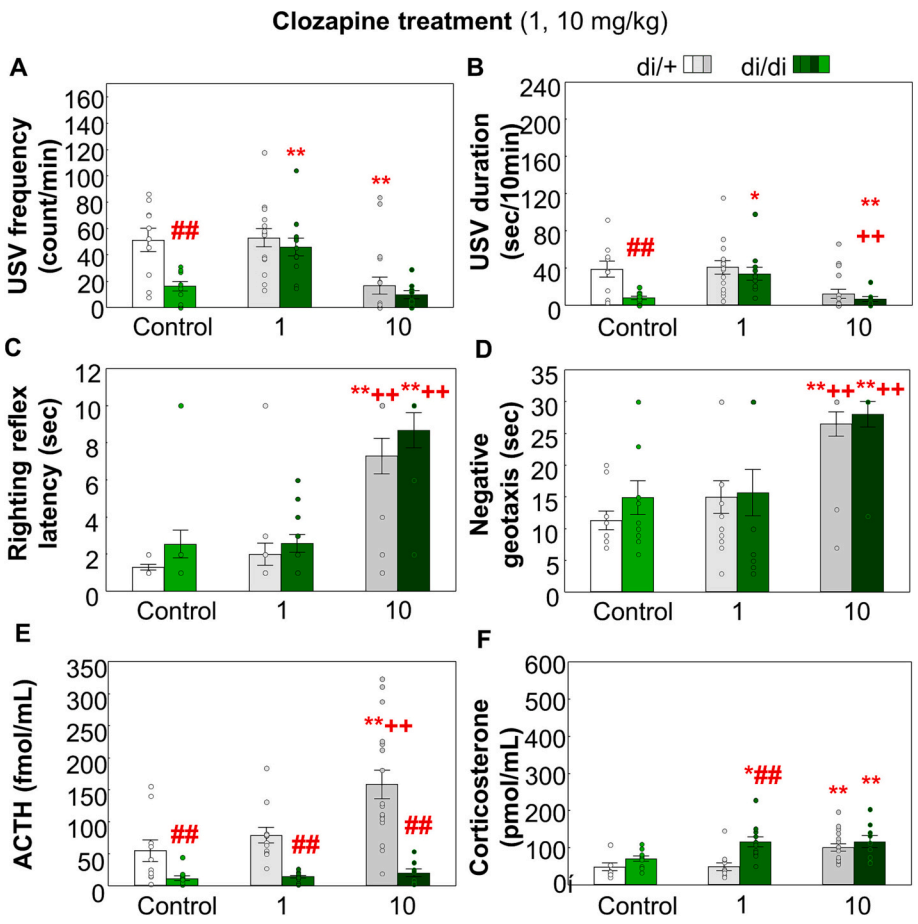

Fig. 3. Clozapine treatment (1, 10 mg/kg). A) USV frequency (count/min) 30 min after treatment; B) USV duration (*sec*/10 min) 30 min after treatment; C) Righting reflex latency (sec) right after MS USV measurement; D) Negative geotaxis (sec) right after MS USV measurement; E) ACTH serum concentration (fmol/mL) after MS USV measurement; F) Corticosterone serum concentration (pmol/mL) after MS USV measurement. ##p < 0.01 significant difference from respective di/+ animals; *p < 0.05, **p < 0.01 significant difference from vehicle treated rats; ++p < 0.01 significant difference from the other treated group.

2.6. Statistical analyses

Data were analysed by two-way ANOVA for factor genotype and treatment using the Statistica 12.0 program of StatSoft, Inc., Tulsa, OK, USA. Post hoc comparison was performed by the Newman– Keuls test. Multiple regression analysis was conducted to determine the contribution of behavioural and hormonal changes to USV. Data are presented as mean ± S.E.M. The level of statistical significance was taken as $p < 0.05$ in all statistical analyses. Main effects are detailed in Table 1., while the results of post hoc comparisons are indicated in the figures with corresponding marks.

3. Results

The weight of di/di pups were significantly lower than that of their di/+ littermates ($p < 0.01$; Table 1.) in all experiments (e.g., haloperidol treatment: di/+: 19.2 ± 0.2 g ($n = 43$); di/di: 16.2 ± 0.2 g ($n = 51$)). The lack of treatment effect confirmed that the animals were evenly distributed among the groups, as body weight was measured before treatment. The genotype had a significant effect on USV frequency and duration in every case ($p \leq 0.01$; Table 1.) with lower levels in di/di pups. The ACTH levels were always significantly lower in di/di rats than in di/+ animals ($p < 0.01$; Table 1.), while corticosterone levels were always higher ($p < 0.01$; Table 1.).

3.1. Haloperidol

Haloperidol treatment had no main effect on MS-USV (frequency: $p = 0.33$, duration: $p = 0.40$). However, in the post hoc analysis, after the 0.1 mg/kg dose the genotype difference both in the USV frequency and duration disappeared, due to a non-significant reduction in the vocalization of the di/+ animals (Fig.2A, B). Behaviour tests did not show significant alterations (Fig.2C, D). Haloperidol dose dependently increased the ACTH ($p < 0.01$) and corticosterone ($p = 0.01$) levels without interaction with the genotype effect (Fig.2E, F).

3.2. Clozapine and olanzapine

The lower doses (1 mg/kg clozapine and 0.3 mg/kg olanzapine) elevated the emitted USVs to the di/+ levels, thus, they were curative for the AVP-deficient rats (treatment effects in all cases for both USV frequency and duration: $p < 0.01$; Fig.3A, B; Fig.4A, B). On the contrary, in the post hoc analysis, higher doses (10 mg/kg clozapine, 3 mg/kg olanzapine) reduced the vocalization of di/+ pups to the di/di level. Nevertheless, the higher, but not the lower doses were sedative on behavioural tests (treatment effects in all cases for both RRL and negative geotaxis: $p < 0.01$; Fig.3C, D; Fig.4C, D) and significantly enhanced the ACTH (p < 0.01; Fig.3E, Fig.4E) and corticosterone (p < 0.01; Fig.3F, Fig.4F) levels. There were no ACTH elevations in di/di animals (genotype x treatment interaction with both (clozapine and olanzapine) treatments: p < 0.01).

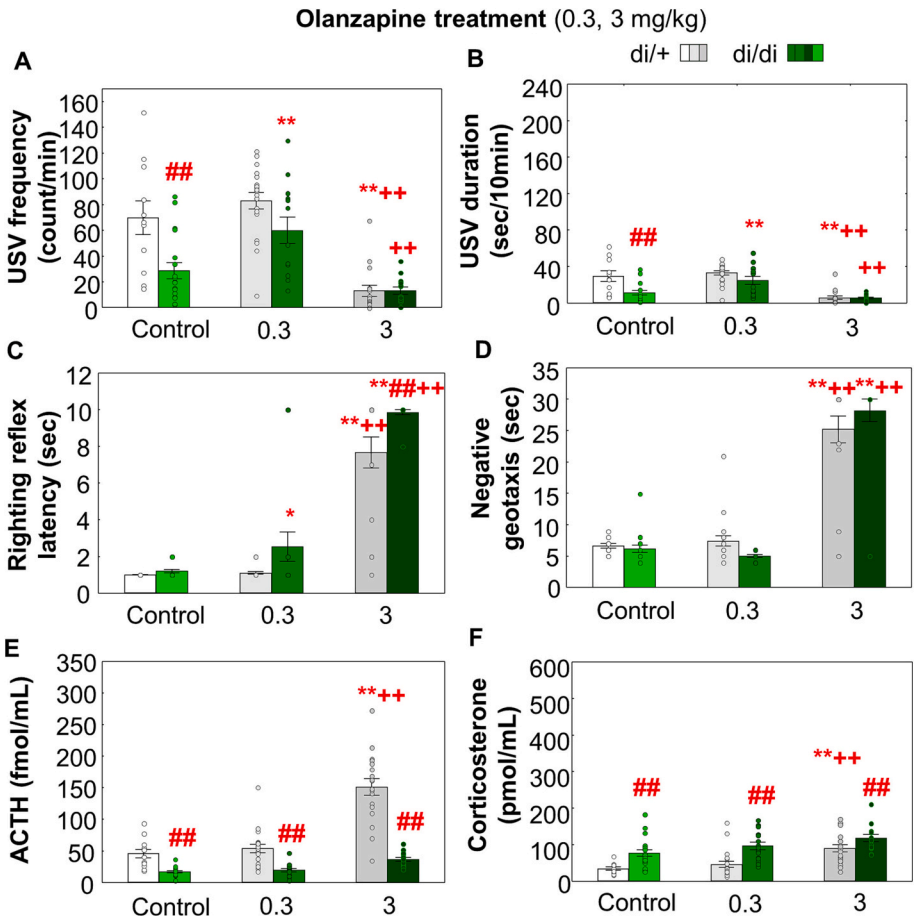

Fig. 4. Olanzapine treatment (0.3, 3 mg/kg). A) USV frequency (count/min) 30 min after treatment; B) USV duration (sec/10 min) 30 min after treatment; C) Righting reflex latency (sec) right after MS USV measurement; D) Negative geotaxis (sec) right after MS USV measurement; E) ACTH serum concentration (fmol/mL) after MS USV measurement; F) Corticosterone serum concentration (pmol/mL) after MS USV measurement. ##p < 0.01 significant difference from respective di/+ animals; *p < 0.05, **p < 0.01 significant difference from vehicle treated rats; ++p < 0.01 significant difference from the other treated group.

Table 2
Results of 0.25 and 1 mg/mg Risperidone treatments.

Genotype	Treatment		USV frequency (count)	USV duration (sec)	Righting reflex (sec)	Negative geotaxis (sec)	ACTH (fmol/mL)	Corticosterone (pmol/mL)
di/+	Control	Average	76.8	82.1	1.0	6.5	24.7	31.4
		SEM	12.3	17.0	0.0	0.7	2.4	5.1
di/di	Control	Average	35.4	30.3	1.5	8.5	6.7	71.4
		SEM	8.8	9.0	0.4	1.7	0.8	8.6
di/+	0.25 mg/kg	Average	34.3	44.2	1.4	7.6	134.1	121.7
		SEM	10.8	17.8	0.2	1.2	9.8	21.0
di/di	0.25 mg/kg	Average	28.1	26.0	1.9	9.5	34.3	217.9
		SEM	5.6	6.5	0.4	1.4	3.0	23.7
di/+	1 mg/kg	Average	16.0	19.7	2.5	20.7	176.8	120.0
		SEM	6.5	9.0	0.6	2.6	10.7	10.9
di/di	1 mg/kg	Average	10.7	8.1	4.7	14.2	41.3	261.0
		SEM	2.6	2.3	0.8	2.4	3.1	25.9

3.3. Risperidone

0.25 mg/kg and 1 mg/kg risperidone dose dependently reduced the emitted USV in both genotypes (frequency $p = 0.39$, duration: $p < 0.01$; Table 2.). At the same time, the higher dose was sedative on behavioural tests (treatment effects for both RRL and negative geotaxis: $p < 0.01$; Table 2.), while even the lower dose increased the ACTH and corticosterone levels (treatment effects in all cases: $p < 0.01$). As we supposed that pronounced HPA-axis activation may override potential pro-communicative drug effects, we repeated the experiment with even lower doses. Indeed, 0.05 mg/kg significantly elevated the frequency and duration of USV in di/di animals (treatment effect, frequency $p = 0.32$, duration: $p = 0.07$; $\text{posthoc}_{\text{control vs 0.05mg/kg}}$ for both frequency and duration: $p < 0.05$; Fig.5A, B). Although none of the tested lower doses (0.05 mg/kg, 0.1 mg/kg) was sedative on behavioural test (treatment for both RRL and negative geotaxis: $p = 0.24$; Fig.5C, D), but even the lowest dose elevated the stress hormone (ACTH and corticosterone) levels (in all cases: $p < 0.01$; Fig.5E, F). AVP-deficient pups showed lower ACTH elevation than their di/+ counterparts with all tested risperidone doses (genotype x treatment interaction: $p < 0.01$).

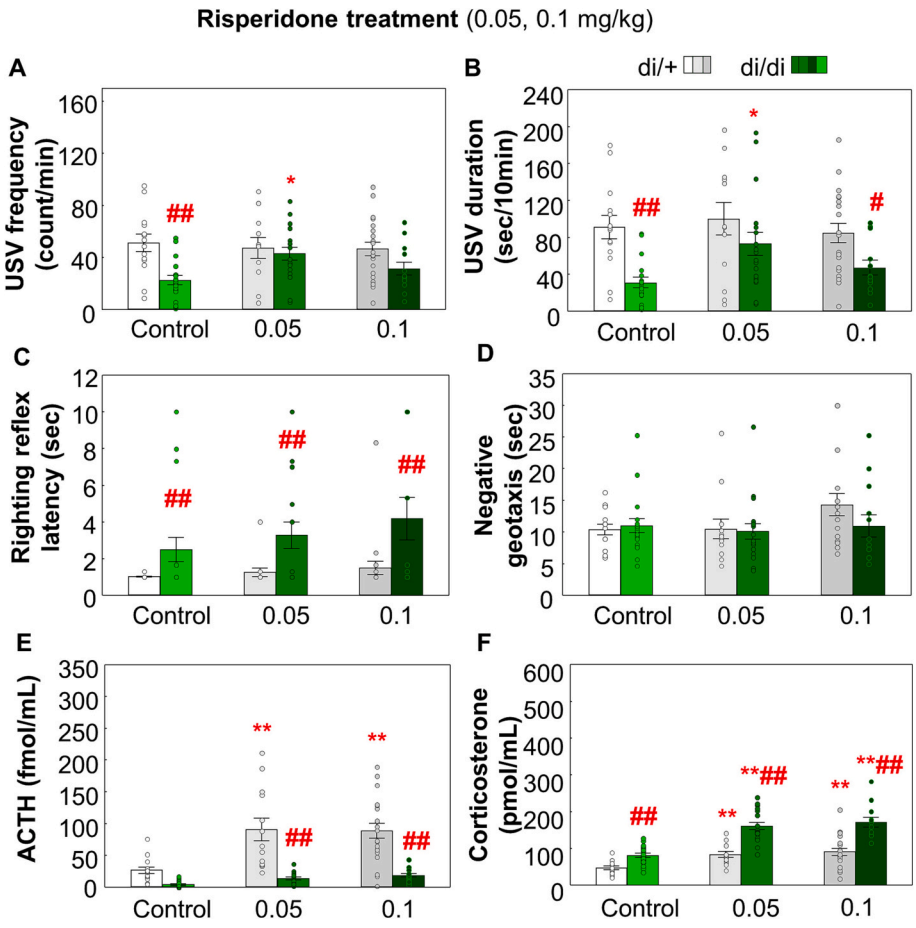

Fig. 5. Risperidone treatment (0.05, 0.1 mg/kg). A) USV frequency (count/min) 30 min after treatment; B) USV duration (sec/10 min) 30 min after treatment; C) Righting reflex latency (sec) right after MS USV measurement; D) Negative geotaxis (sec) right after MS USV measurement; E) ACTH serum concentration (fmol/mL) after MS USV measurement; F) Corticosterone serum concentration (pmol/mL) after MS USV measurement. #p < 0.05; ##p < 0.01 significant difference from respective di/+ animals; *p < 0.05, **p < 0.01 significant difference from vehicle treated rats.

3.4. Aripiprazole

Aripiprazole in 0.5 mg/kg and 5 mg/kg doses significantly elevated the USV frequency and duration in di/di pups (treatment effect, frequency p < 0.01, duration: p = 0.01; Fig.6A, B). Neither dose was sedative on behavioural tests (treatment for RRL: p < 0.01 due to a reduction, not elevation after the 0.5 mg/kg; negative geotaxis: $p = 0.75$; Fig.6C, D). However, there was an elevation of righting latency in some di/di animals (genotype effect: p < 0.01), without changes in negative geotaxis. The 5 mg/kg aripiprazole dose significantly elevated serum ACTH levels in all animals (p < 0.01) with lower levels in di/di pups (genotype x treatment interaction: p < 0.01; Fig.6E). A remarkably high dose (50 mg/kg) could not "normalized" the USV (treatment effect, frequency $p = 0.96$, duration: $p = 0.79$; Fig.6A, B), had mild sedative side effect (treatment for RRL: $p = 0.14$; negative geotaxis: $p = 0.016$; genotype x treatment interaction for negative geotaxis: $p = 0.027$) observable only in di/+ animals, with a significant elevation in ACTH concentrations (p < 0.01).

4. Discussion

We could confirm our hypothesis that the reduced USV of AVP-deficient rats is indeed a sign of disturbed communication which can be restored by low, non-sedative doses of atypical antipsychotics.

Our research is the first which examined the effect of antipsychotics on MS-USV on such a wide scale. Only haloperidol-related studies can be found in the literature. Pretreatment with haloperidol reversed the cocaine-induced decrease in USV in 10-day old rat pups (Dastur et al., 1999; Kehoe and Boylan, 1992). However, in our completely different model (i.e., different age and maternal separation), haloperidol could not normalize the USV deficit of the Brattleboro rats. Cocaine affects dopamine system that regulates reinforcement and reward (Kehoe and Boylan, 1992), which basically means ventral tegmental area dopaminergic neurons. In AVP-deficient rats, however, it is hypothesized that the most marked effect of AVP deficiency on the dopamine system is in the amygdala: AVP is a potent enhancer of dopamine utilization in that brain region (van Heuven-Nolsen et al., 1984). Another study suggests that dopamine D1 and D2 receptors may differentially modulate USV emission (Dastur et al., 1999): the selective D2 receptor antagonist (sulpiride) decreased USV rate of rat pups, but the D2/D3 receptor antagonist (raclopride) and the selective D1 receptor antagonist (SCH 23390) had no effect on it. Our results are similar to those found with raclopride and SCH 23390. This may be explained by the fact that haloperidol is an antagonist of all three dopamine receptors mentioned (Siafis et al., 2018). It was also found that high doses of three dopamine receptor agonists (D1, D2/D3, D3) reduced USV (Dastur et al., 1999), which supports an important role of the dopamine system in its regulation.

Lower doses of clozapine, olanzapine, risperidone and aripiprazole restored MS-USV in our study. Antipsychotic drugs (in these doses) do not have anxiogenic effects, furthermore, there are data for their anxiolytic effects (Biojone et al., 2011; Manzaneque et al., 2002). Thus, we might rule out the possibility that the elevation of the USV was because of the elevated anxiety level of the treated pups, rather this may be

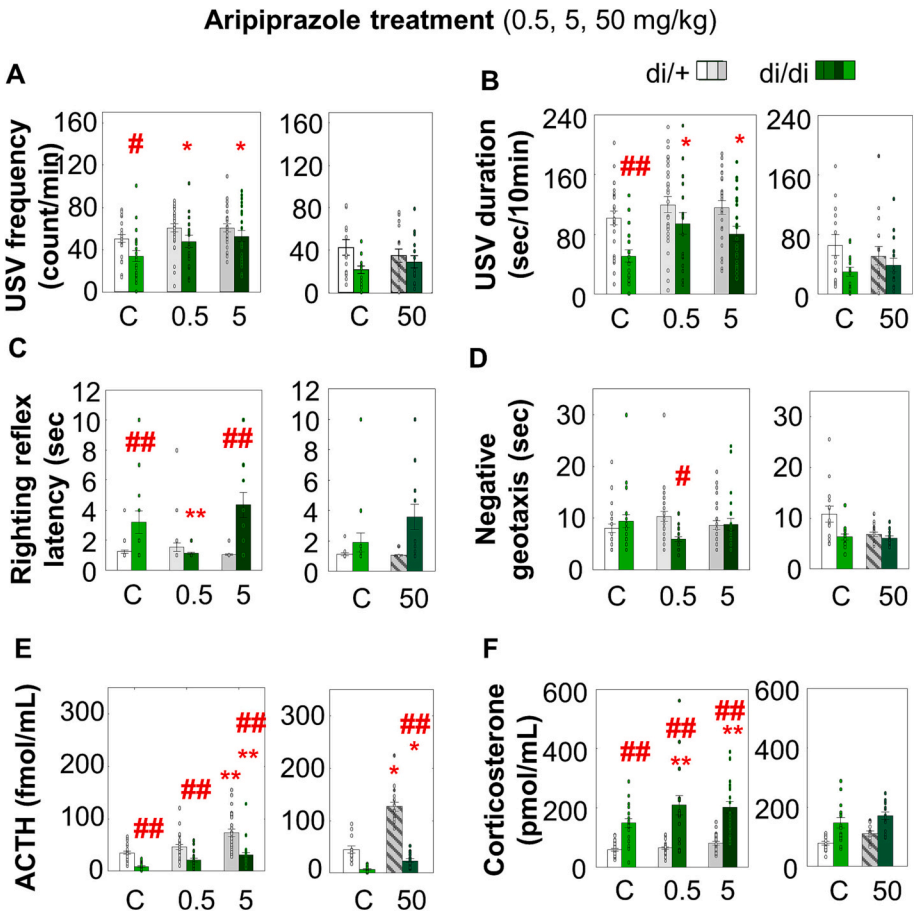

Fig. 6. Aripiprazole treatment (0.5, 5, 50 mg/kg). A) USV frequency (count/min) 30 min after treatment; B) USV duration (sec/10 min) 30 min after treatment; C) Righting reflex latency (sec) right after MS USV measurement; D) Negative geotaxis (sec) right after MS USV measurement; E) ACTH serum concentration (fmol/mL) after MS USV measurement; F) Corticosterone serum concentration (pmol/mL) after MS USV measurement. #$p < 0.05$, ##$p < 0.01$ significant difference from respective di/+ animals; *$p < 0.05$, **$p < 0.01$ significant difference from vehicle treated rats.

related to the improvement of the communication deficit.

The abovementioned atypical antipsychotics share properties that haloperidol does not, particularly a much stronger action on serotonin receptors. There are experiments suggesting that USV is under control of the serotoninergic system (Iijima and Chaki, 2005; Olivier et al., 1998). The effects of the examined antipsychotics on serotonin receptors are highly complex (Siafis et al., 2018), and their impact on USV has not yet been investigated. It has been reported that 5-HT1A receptor agonist decreases MS-USV in rat pups (Groenink et al., 2008). Additionally, the potent 5-HT7 receptor antagonist amisulpride improved ketamine-induced social withdrawal in rats (Holuj et al., 2015). Therefore, it may be hypothesized that 5-HT1A and 5-HT7 receptors play a role in social communication.

Regarding stress hormone levels in our study, stressor-induced ACTH levels were lower, whereas corticosterone levels were higher in di/di rats compared with controls. The functional activity of the HPA axis is reduced in homozygous Brattleboro rats, as reflected by markedly decreased ACTH concentrations in both plasma and the adenohypophysis, accompanied by reduced adrenal weight (Buckingham and Leach, 1980). The role of AVP is important in the ACTH secretion regulation during most acute stimuli, but the contribution of AVP to stress-induced HPA activity is context dependent (Makara et al., 2012). In our previous study we confirmed the hypothesis that during the perinatal period, AVP may be the main regulator of the HPA axis (Makara et al., 2012; Zelena et al., 2009; Zelena et al., 2015): stressors-induced ACTH rise was always smaller in di/di pups, than in their di/+ littermates. Furthermore, the resting corticosterone levels of di/di pups were higher. This phenomenon might have relevance also in humans: although there is no evidence that AVP is the main regulator of HPA axis during early development, higher blood levels of cortisol were found in male ASD children compared with healthy controls (Bozkurt et al., 2021): a meta-analysis with 375 ASD patients and 335 controls also supported this (Gao et al., 2022). Interestingly, a 2003 study found lower serum cortisol and higher ACTH levels in children with ASD (Curin et al., 2003). Another measurement detected higher ACTH as well as cortisol levels (Iwata et al., 2011), however, Bouvard et al. published abnormally low level of ACTH in ASD (Bouvard et al., 1995). Mainly in the ACTH level, there are conflicting results in the literature: this can even be explained by the difficulty of the measurement (Gibson et al., 1989), or it is possible that the ACTH level varies in each autism subtype or spectrum, but a comprehensive study on this does not yet published.

In our present study haloperidol, risperidone and aripiprazole increased ACTH and corticosterone levels (clozapine and olanzapine also, but only at the high, sedative doses). A human study had shown that haloperidol administration can increase cortisol levels (Murburg et al., 1986), but basically most clinical studies have found that antipsychotics reduce stress hormone levels or do not influence them (Handley et al., 2016; Piriu et al., 2015). However, there is no such data for perinatal age, thus, our finding might be specific to this age group.

Nevertheless, the hypothesis that HPA-axis activation drives reductions in MS-USVs—particularly at high risperidone doses—appears to contradict evidence that anxiety can increase USVs (Groenink et al., 2008), especially considering that prolonged glucocorticoid elevation can induce anxiety (Ardayfio and Kim, 2006; Shoji et al., 2024).

However, infant vocalizations may attract not only the dam but also predators, creating natural selective pressure for silencing isolated pups, particularly under high-stress conditions. Supporting this idea, contact with unfamiliar adult male rats increases ACTH while simultaneously suppressing isolated pup calling (Hofer, 1996). Likewise, intracerebroventricular administration of corticotropin-releasing hormone, a central regulator of the HPA axis, reduces MS-USVs under highly stressful hypothermic conditions (Dirks et al., 2002). Accordingly, drug-induced HPA-axis activation may override potential pro-communicative effects of the treatment, resulting in an overall reduction of MS-USVs.

We previously demonstrated that MS-USV emission does not differ between heterozygous and homozygous wild-type Brattleboro animals (Varga et al., 2015), which justifies the use of heterozygous animals as controls in the present study. Nevertheless, we cannot entirely exclude the possibility that heterozygous animals respond differently to antipsychotic treatment compared with homozygous wild-type controls, particularly given the lack of available data in the literature on the effects of antipsychotics on MS-USVs.

5. Conclusion

Overall, this study supports the hypothesis that AVP-deficient Brattleboro rat pups represent a useful model for studying impairments in early-life social communication, which may also be highly relevant in the context of ASD, where early deficits in social communication represent a core diagnostic feature. The ability of pharmacological interventions to modulate these deficits suggests that MS-USVs capture a biologically meaningful aspect of early communicative function. Importantly, the paradigm requires only minimal drug exposure and is applicable to a broad range of compounds, including non-antipsychotic and neuromodulatory agents, making MS-USV deficits in Brattleboro pups a valuable tool for mechanistic studies and preclinical screening of communication-targeting interventions.

CRediT authorship contribution statement

Bibiána Török: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Anna Fodor:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Barbara Klausz:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **János Varga:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dóra Zelena:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Compliance with ethical standards

The study was conducted according to the guidelines of the Declaration of Helsinki, in accordance with the European Communities Council Directive recommendations for the care and use of laboratory animals (2010/63/EU) and was approved by the Animal Welfare and Ethics Committee of Institute of Experimental Medicine (22.1/3895/003/2009 and PEI/001/38-4/2013). The authors complied with the ARRIVE guidelines.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2026.111682.

Data availability

The data are available upon reasonable request.

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