Sex differences in ultrasonic vocalization and hormonal stress response of an anxious mice strain during the early postnatal period

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Abstract. Development of anxiolytics requires animal models; therefore anxious (AX) and nonanxious (nAX) mice have been selectively bred based upon their adult behaviour in reaction to handling. Since inadequate response to postnatal challenges may have lifelong consequences, we aimed to determine whether alterations in postnatal stress-coping may contribute to the state of anxiety in adult AX mice. Maternal separation-induced ultrasonic vocalization (MS-USV) was studied in one-week-old nAX-AX mice and one week later the endocrine stress response was measured after lipopolysaccharide (LPS) stimulation, a model of bacterial infection, with special focus on sex differences. It was established that AX females produced a significantly lower frequency and duration of MS-USV than nAX female mice. USV emitted by AX males showed only a tendency to be reduced compared to nAX males. LPS injection generated a significantly increased adrenocorticotropin release in nAX mice only and with no sex-related difference observed. Resting corticosterone levels were higher in AX mice compared to nAX, however, this difference reached the level of significance only in females. LPS-injection was able to induce significant corticosterone elevation with markedly higher levels in AX females than nAX females. To sum up, we conclude that females are more susceptible to the influence of stressors during the early postnatal period, as they exhibited more pronounced reactions. The adult state anxiety of the strain was not paralleled by MS-USV during the perinatal period. Thus, the question arises if MS-USV indeed reflects anxiety only. According to our expectation, AX animals had higher baseline stress-hormone levels, but their reactivity to stressors was altered, which may have contributed to their supposed adult phenotype.

Keywords: adrenocorticotropin, corticosterone, lipopolysaccharide, pup, ultrasonic vocalization.
Introduction

Pathological anxiety is quite common not only in developed, but also in developing countries, and its consequences lie heavy upon society (Kessler et al. 2009). Although animals can merely mimic the complex anxiety-related behaviour observable in humans, the low genetic variability of inbred rodent strains as well as easy manipulation and availability of tissues make animal models attractive. To study the mechanisms and develop new treatments, anxious mice (AX) were selected on the basis of anticipatory anxiety behaviour during a handling procedure (Szegő et al. 2010; Horváth et al. 2013). After several generations, AX mice were more anxious compared to controls (nAX) in different well-established anxiety tests, such as the open field, elevated plus maze, and light dark tests.

Although a difference in the reaction to early-life adversity, such as suboptimal maternal care or exposure to stress, may program the brain differentially and lead to different adult phenotype (Horváth et al. 2013; Maccari et al. 2014; Maniam et al. 2014), little is known about the postnatal reactivity of inbred anxious models and its contribution to the development of their adult anxiety. In this paper we aim to clarify this issue.

Separation from the nest is a distress that induces the activation of hypothalamic-pituitary-adrenocortical (HPA) axis in the offspring (Varga et al. 2015). Corticotropin-releasing hormone and arginine vasopressin from the hypothalamus control the adrenocorticotropin (ACTH) secretion in the adenohypophysis, which is the main regulator of glucocorticoid (in rodent corticosterone) release in the adrenal cortex (Ise et al. 2008; Walker et al. 2009). The behavioural consequence of maternal separation is the emission of ultrasonic vocalisation (USV) by the pups. Various animal species use USV to communicate with each other and provide information about the environment (Brudzynski 2018). The quality and quantity of emitted USV changes with age (Portfors 2007). Pups in stressful situations use ultrasonic vocalization to call their mothers. The frequency and duration of such USV are considered indicators of anxiety, as anxiolytics can attenuate them (Iijima, Chaki 2005), making USV a widely used test of putative anxiolytic drugs.

Infections during the perinatal period are rather common and may contribute to the life-long programming of the brain and behaviour (Nguyen et al. 2015). The adaptability to challenges can be measured by changes in the hormones of the HPA axis. Indeed, lipopolysaccharide (LPS), a section of a Gram negative bacteria wall, induces a severe stress reaction in vulnerable individuals (Lolait et al. 2007; Salome et al. 2008; Walker et al. 2009; Balázsfi et al. 2016).

In the present research we aimed to study maternal separation-induced USV and stress-hormone reactivity to LPS injection in AX and nAX mice pups in order to establish whether their altered behavioural and hormonal stress-sensitivity during the challenges of the postnatal period may contribute to the development of psychopathologies in this strain.

Materials and methods

1. Animals

nAX and AX inbred mouse strains were originally developed at EGIS Pharmaceuticals Co. (Budapest, Hungary) by combination of crossbreeding and behavioural selection (Szegő et al. 2010). Selection was made in adult mice on the basis of anticipatory anxiety behaviour during the handling procedure. When mice were moved to another cage, some animals always ‘volunteered’ earlier, while others always moved away from the experimenter. Animals were separated into two groups, early movers (non-anxious, nAX, control) and late movers (anxious, AX), and were inbred.

Pups were kept with their mothers under a standard 12h light–dark cycle (lights on at 8 a.m.), with food and water available ad libitum, and their body weights were recorded during the tests. All experiments were carried out between 9h and 14h. All manipulations with the animals were approved by the local committee for animal health and care and performed according to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2. Experimental design

On postnatal day (PND) 7–8 (both sexes, 10 litters, 6–10 pups/litter) pups were examined in an ultrasonic vocalization test (USV, see details below; n = 14–19/group), marked and placed back with their mothers. A week later (PND 14–15) LPS (Sigma-Aldrich, St. Louis, Mo. USA; O55:B5; 100 µg/ml/kg, dissolved in saline) or saline was injected intraperitoneally (i.p.) to the same animals (n = 5–9/group) (Balázsfi et al. 2016). One hour later blood samples were taken for hormone measurements (ACTH, corticosterone).

3. Ultrasonic vocalization (USV)

Pups were brought to a soundproof room and placed in a 600 ml glass beaker without bedding and heating. Several studies demonstrated that ambient temperature plays a pivotal role in the rates of separation-induced vocalization. USV is lower when the temperature is close to nest or skin temperature (30–37 °C). In contrast, USV rates are higher at room temperature (Portfors 2007; Ise et al. 2008;
Walker et al. 2009). We chose the more stressful room temperature condition for our experiments.

USV was observed for 10 min. as described in one of the previous studies (Varga et al. 2015). To describe the procedure briefly, individual calls were detected by means of an ultrasonic-sensitive frequency division detector (CIEL electronique, CDB205 R2, coupled to a computer), which was fixed on a holder 12 cm above the bottom of the glass beaker. Vocalizations were recorded using free Audacity 2.0.5. software and stored on a personal computer. Data were automatically counted in the power spectrum 30–50 kHz (typically occurring after maternal separation (Varga et al. 2015)) using a USV Counter software (developed by S. Zsebők). The total number and total duration of calls per session were measured. In addition, USV frequency was calculated as the total number /10 min.

4. Hormone measurements

Blood was collected after decapitation in ice-cold plastic tubes, it was centrifuged, and the serum was separated and stored at –20°C until analysis. ACTH and corticosterone were measured by radioimmunoassay in 50 μl or 10 μl unextracted serum, respectively, as described in earlier research (Varga et al. 2015). The intra-assay coefficients of the variations were 4.7% and 7.5% for the two hormones, respectively. All the samples from a particular experiment were measured in one radioimmunoassay.

5. Statistical analysis

Data were expressed as means ± SEM and analyzed using STATISTICA 12.0 software package (StatSoft, Inc., Tulsa, OK, USA) by analysis of variance (ANOVA) using repeated measure (body weight; factors “phenotype”, “time” and “sex”), two (USV; factors “phenotype” and “sex”) or three way ANOVA (LPS; factors “treatment”, “phenotype”, and “sex”). Post-hoc comparison was made by the Newman-Keuls method and the results were presented in figures (shown below). Correlation was calculated by the Pearson analysis. P values under 0.05 were considered as significant and between 0.1 and 0.05 was considered as trends.

### Results

1. Weight

At 7–8 PND the body weight of all groups was homogenous (Table 1). However, 7 days later, at the time of LPS injection the AX females were significantly heavier compared both to nAX females and AX males (phenotype x sex: $F_{1,55} = 3.74, p = 0.05$; phenotype x time x sex: $F_{1,55} = 3.19, p = 0.07$).

2. Anxiety-like behaviour (USV)

Frequency (number of calls/10 min.) (phenotype: $F_{1,62} = 8.02; p < 0.01$) as well as duration (phenotype: $F_{1,62} = 11.09; p < 0.01$) of vocalization were generally lower in AX compared to nAX animals, but during post-hoc comparisons the difference reached the level of significance only in females (fig. 1). Thus, the AX mice emitted significantly less USV both in frequency (number of calls per minute; A, B) and duration (added together during the entire 10 min. observation period; C, D) than nAX mice, which reached the level of significance in females only. * $p < 0.05$, ** $p < 0.01$ vs nAX

Table 1. Body weight of the animals

| Weight (g) | nAX Male | nAX Female | AX Male | AX Female |
|------------|----------|------------|---------|-----------|
| PND 7      | 4.26 ± 0.14 | 4.08 ± 0.15 | 4.63 ± 0.23 | 4.62 ± 0.25 |
| PND 14     | 7.46 ± 0.23 | 6.87 ± 0.19 | 6.99 ± 0.47 | 7.91 ± 0.44*# |
| n          | 19       | 19         | 15      | 14        |

* $p < 0.05$ vs female nAX; # $p < 0.05$ vs male AX

![Fig. 1. Maternal separation-induced ultrasonic vocalization (USV) on postnatal day (PND) 7–8 in anxious (AX) (n = 19 male and 19 female) and nonanxious (nAX) mice (n = 15 male and 13 female). AX mice emitted significantly less USV both in frequency (number of calls per minute; A, B) and duration (added together during the entire 10 min. observation period; C, D) than nAX mice, which reached the level of significance in females only. * $p < 0.05$, ** $p < 0.01$ vs nAX.](image-url)
maternal separation resulted in sex-dependent differences during the USV test.

3. Stressor exposure (LPS)

One hour after LPS injection a robust elevation of plasma ACTH was observed in nAX, but not in AX mice (treatment: $F_{(1,48)} = 37.7; p < 0.01$; phenotype x treatment: $F_{(1,48)} = 22.8; p < 0.05$). ACTH levels did not show sex-dependent changes either under control conditions, or after LPS injection (fig. 2A, B).

Plasma corticosterone levels after LPS treatment increased in both phenotypes (treatment: $F_{(1,44)} = 4.81; p < 0.05$). AX animals showed a higher level of corticosterone both under basal conditions and after stressor exposure than nAX ones (phenotype: $F_{(1,44)} = 33.2; p < 0.01$). During post-hoc comparison this hormonal hyperactivation in AX pups was significant only in females (fig. 2C, D).

Finally, we found a significant negative correlation between the studied USV parameters and corticosterone levels among control conditions (USV frequency — corticosterone: $r = -0.45, p < 0.05$; USV duration — corticosterone: $r = -0.46, p < 0.05$). There were similar tendencies after stressor exposure also, but it did not reach the level of significance (USV frequency — corticosterone: $r = -0.35, p = 0.08$; USV duration — corticosterone: $r = -0.38; p = 0.06$).

Discussion

Our findings demonstrated that nAX animals emitted significantly higher USV after maternal separation than AX pups. This difference was also more pronounced in females than males. Acute immune stress had a prominent effect on ACTH response in nAX pups only, whereas corticosterone levels in AX mice appeared constantly higher both in basal and stress conditions.

Previous studies clearly suggested an enhanced maternal separation-induced USV emission as a sign of exaggerated anxiety both in rat and mice pups (Winslow, Insel 1991; Iijima, Chaki 2005; Varga et al. 2015; Balázsfi et al. 2016). Moreover, rats showing higher USV at PND 10 during a 2 min test were more anxious in their adulthood as measured in open field (Brunelli, Hofer 2007). However, we have to emphasize, that their “controls” with low USV during the perinatal period seemed to be more aggressive and emit lower 55 kHz (pro-social, Fig. 2. Changes in stress-hormone levels 60 minutes after intraperitoneal lipopolysaccharide (LPS, 100 μg/ml/kg) injection on postnatal day (PND) 14–15 in neonatal anxious (AX) and non-anxious (nAX) mice (n = 8, 4, 6, 8 for males and 8, 5, 4, 9 for females, in the order of the columns): (A, B) adrenocorticotropin (ACTH, fmol/ml) levels increased in response to LPS injection only in nAX mice without sex-dependent differences; (C, D) corticosterone (pmol/ml) levels were higher in AX mice both during rest and after LPS stimulation. LPS induced significant elevation in both phenotypes. The AX females showed higher levels than their nAX counterparts. * $p < 0.05$ vs nAX, ** $p < 0.01$ vs saline

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marker of positive effects) USV in adulthood, thus, the adult anxiety in these animals was not unequivocal. Indeed, another laboratory found a negative correlation between infant anxiety behaviour examined by maternal separation-induced USV for 10 min. and adult anxiety measured as immobility and 22 kHz USV during fear conditioning (and partly by enhanced open arm frequency on elevated plus maze in high infantile USV group compared to low USV rats; p = 0.058) (Wöhr, Schwarting 2008). We agree with the authors that the emission of ultrasonic calls in infancy might reflect an active coping style, based upon positive correlations between calling and overt behaviour observed by them, while in adulthood USV might reflect a passive coping style shown by the same group with a positive correlation between calling and immobility. Moreover, behavioural effects observed during isolation (e. g. USV) are part of stress response, while behaviours during testing in novel, but unthreatening situations (e. g. handling as a selection method in our strain or elevated plus maze) reflect the need for exploration. However, in contrast to our findings, another inbred strain, the high anxiety behaviour (HAB) and low anxiety behaviour (LAB) animals selected based upon their behaviour on elevated plus maze, was found to emit higher USV examined either in 11-day-old rat pups (Wigger et al. 2001), or 5-day-old mice pups (Krömer et al. 2005), or adult rats in response to social defeat (Frank et al. 2006). We might assume that different factors contribute to the anxiety phenotypes in the two anxious strains.

Maternal behaviour has a lasting consequence on the adult anxiety-related behaviour and coping style (Maniam et al. 2014; Nguyen et al. 2015). It was shown that maternal licking and grooming induce certain epigenetic changes in the pups, resulting in altered stress responses and reduced anxiety in adulthood (Weaver 2007; Lester et al. 2018). Increased USV in nAX pups might induce more active maternal care, which might in turn contribute to the development of a non-anxious adult phenotype. On the contrary, the reduced USV in AX pups might not promote sufficient maternal nurturing, affecting the later-life behaviour. We intend to explore the level of maternal care in our mouse strain in a follow-up study.

The higher resting stress hormone levels in AX mice pups may also result in negative consequences later (Musazzi, Marrocco 2016). Moreover, the reduced stress reactivity to an immune challenge in AX mice pups is also a reflection of a disrupted coping style. Previously LPS was able to induce significant stimulation in ACTH and corticosterone levels in adult (Lolait et al. 2007) and postnatal (Balázsfi et al. 2016) mice, which was replicated in our present experiments. Although HAB rats previously demonstrated a larger increase in corticosterone levels to LPS administration than LAB rats (Salome et al. 2008), their stress reaction to social defeat was also diminished (Frank et al. 2006), similarly to our results. In our case, the lower level of reactivity might be explained by the already enhanced basal stress hormone levels.

Our data confirms the findings of a previous study in a mutant mice strain, which suggested sex-determined differences, particularly a higher stress-hormone release in female subjects compared to males (Lolait et al. 2007), thus supporting the idea of increased vulnerability of females in response to an acute stressor. Moreover, a previous study in C57BL/6J mice found female pups to be more susceptible than males to the effect of maternal separation based on USV call frequency and duration (Yin et al. 2016). In our experiment nAX females showed a tendency towards higher USV than males (p = 0.08) while there was no marked sex-related difference observed in the response of AX mice, and this could mean a significantly different rate of USV when comparing AX to nAX in females, but not in males.

**Conclusion**

According to the three-hit concept of vulnerability and resilience, aside from genetic predisposition and stressful experiences in adulthood, the early perinatal period is of utmost importance (Daska-lakis et al. 2013). We might conclude that — in accordance with our expectation — the higher basal corticosterone levels of AX animals and their altered stress reactivity to immune challenges may contribute to their adult phenotype. The adult anxiety phenotype of the strain was not paralleled by higher maternal separation-induced USV, thus, the question arises whether USV is indeed a good model of perinatal anxiety or if it rather resembles social communication or active coping with stress.

**Conflict of interest statement**

No potential conflict of interest relevant to this article was reported.

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