Strain- and context-based 50 kHz ultrasonic vocalizations and anxiety behaviour in the Wistar-Kyoto rat

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Rodent ultrasonic vocalizations (USVs) are influenced by immediate, prior contexts and have emerged as important indicators that monitor an individual's 'state'. They also index direct reflections of inherent 'trait' and are suggested to constitute non-invasive read-outs of pathological conditions. Analysis of USVs emitted under particular contexts could help discern strain-specific differences and existence of individual USV profiles. USVs of the Wistar-Kyoto (WKY) strain, a putative model of depression, could indicate social communication deficits. In the cage, USV emission was significantly reduced in WKYs. An elevated plus maze exposure led to no change in USV emission in WKYs, while it significantly reduced USVs in Wistars. Re-exposure induced strain-specific differences in behaviour and total calling time. Sonographic patterns indicated that the predominant USV subtype were flat 50 kHz USVs. EPM exposure induced a reduction in peak amplitude in WKY USVs and in USV length in both strains. USV peak frequency and amplitude, genetically determined spectral features, were strain-specific, while bandwidth and temporal features such as total calling time and USV duration were context-dependent. WKY USVs demonstrated characteristic spectral structures such as increased call length and reduced peak frequency while other parameters were not quantitatively different, reflecting the shared phylogeny between Wistars and WKYs.

1. Introduction

Rodent USV emissions are said to constitute a communication model that manifests the emotional/motivational aspects regarding the animal's social state/context (Burgdorf and Moskal 2010). Based on their rate, duration, amplitude and modulation, they can be distinguished as reflecting positive or negative affective states (Panksepp 2010). Analysis of this behavioural response could prove useful to characterize communication between pups and dam, adolescents, and adults of the same or opposite sex. USV emissions in various rat strains, such as Wistar (Schwarting *et al.* 2007), Sprague Dawley (Willey *et al.* 2009; Willey and Spear 2012) and Long Evans (Wright *et al.* 2010), have been analysed, although no report exists as yet on the adult Wistar-Kyoto rat (WKY). The WKY, which was generated as a normotensive

control for the spontaneously hypertensive rat (Sterley *et al.* 2011), is being suggested as an endogenous model of depression (O'Neil and Moore 2003; Will *et al.* 2003).

Pup and adolescent USV emissions in the WKY have been studied: WKY pups demonstrate less separation-induced USVs and proximity-seeking behaviours, indicative of withdrawal symptoms when exposed to mild chronic stress (Braw *et al.* 2008). General inactivity in WKYs in the face of novelty is said to be characteristic of their reserved response, reflecting low basal levels of serotonin (Howells *et al.* 2009). Earlier, we have shown that serotonin agonists can induce or reduce USV production to varied extents (Sadananda *et al.* 2012). As administration of anxiolytics influences USV production, they could also constitute non-invasive read-outs of pathological states (Kaltwasser 1991). The impaired adaptive capability is said to make WKYs more susceptible to depressive

behaviours. They do not show habituation (Jiao *et al.* 2011), demonstrate learned helplessness, and are prone to develop stress-induced anxiety-like characteristics (McAuley *et al.* 2009). Such a behavioural phenotype should manifest in variations in USV parameters, whether rate, type and pattern, when compared to Wistars.

Based on spectral features, three broad USV types have been identified which vary depending on age and environmental conditions. Of these, the 50 kHz USVs are heterogeneous, comprise flat or constant frequency USVs and frequency-modulated USVs (Wright *et al.* 2010), have a frequency at peak energies of 32–96 kHz, and are mostly of narrow (1-7 kHz) bandwidths and shorter duration (30–50 ms). They show varying degrees of frequency modulation with considerable variation in spectrographic structure. Overt behaviours associated with these include approach and increased exploratory behaviour (Sadananda *et al.* 2008).

Our earlier research has focused on the possible biological functions contained in these signals, and whether certain situations/contexts promote their emission. Rats exposed to a test arena or a cage with fresh bedding emit USVs (Sadananda *et al.* 2012) with the presence of bedding potentiating amphetamine-induced USV rates (Natusch and Schwarting 2010). USVs thus constitute indicators of the actual presence of desirable and/or social stimuli and are also emitted in anticipation thereof, for instance, in re-establishing contact with the cage mate. Detailed spectrographic analysis of other quantitative and qualitative parameters could prove useful in understanding the communicative value of these USVs.

Here, WKY and Wistar rats were tested in a cage with bedding and in an elevated plus maze (EPM). The EPM consisted of two closed and two open arms, and is said to constitute a novel, anxiogenic context that assesses the conflict between approach and avoidance. Both strains were exposed to the same EPM again after an interval to ascertain changes in USVs rate, temporal and acoustic profiles. The EPM has the typical 'one trial tolerance effect' for behavioural measures. The repeated exposure here was to detect whether USV emission is in line with the suggested passive coping style of WKYs. A low level of USVs irrespective of the context, with no or very little habituation, and a profile that is different from Wistars is to be expected. The occurrence of a temporal pattern in these USVs could provide more insights into strain- and context-specific emission of USVs.

2. Methods

2.1 *Animals*

Naïve adult male Wistar and WKY (n=20 each) rats weighing 210.46±4.46 g were procured from the National Centre for Laboratory Animal Sciences, National Institute of Nutrition (NCLAS-NIN), Hyderabad, India, and were housed in groups of four under standard laboratory conditions with food and water provided *ad libitum*. All animals were handled on 3 consecutive days before the experiments began. The housing room was maintained on a 12 h LD cycle with ambient temperature of 25–27°C. All experiments were conducted in the light cycle (9:00–17:00 h), and in accordance with the ethical regulations for animal experimentation laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and cleared by the Institutional Animal Ethics Committee.

2.2 *Cage test*

Animals were tested individually in a separate experimental room. They were removed from their home cages and tested individually in a fresh cage with bedding in the adjacent room. Cages were placed ~50 cm above the ground. Video recording started immediately thereafter.

2.3 *Elevated plus maze*

Twenty-four hours later, the animals were placed in a cleaned EPM (EPM-1) that was elevated 50 cms above the floor, was of black acrylic and consisted of two open arms (50×10 cm) and two closed arms with no roof (50×10×40 cm) at right angles to each other and an open square (10×10 cm) in the centre. The animals were placed in the centre, always facing the same open arm. USV and behaviour recordings were carried out simultaneously and began as soon as the rat was placed in the centre of the maze. The maze was cleaned thoroughly before each animal was put in. Eight days later, a repeat exposure to the same EPM (EPM-2) under similar testing conditions was carried out.

2.4 *Behaviour recording and analysis*

Video recording was done using a ceiling-mounted Panasonic WV CP500 CCD camera fed to a Piccolo frame grabber card and analyzed using Ethovision XT version 8.0® (Noldus, Netherlands). All recordings were done for 5 min under 8–8.5 lx light intensity. EPM parameters: open and closed arm time and entries, centre time, distance moved and latency to enter the open arm were scored automatically after validation by double-blind manual scoring. Rearing and dipping frequencies were scored by a trained person double-blind to the experimental design and animal genotype.

2.5 USV recording and analysis

USVs were recorded as described in detail elsewhere (Sadananda *et al.* 2008, 2012). For the analysis, 50 kHz USV detection was done interactively by an experienced user blind to the experimental design, who marked them manually from start to end before the system analyzed them. A total of 312 Wistar USVs and 370 WKY USVs emitted in different contexts were marked in this manner for further analysis. Display was set such that the USVs could be distinguished from the background. The following parameters were obtained: Mean peak frequency (frequency at the location of the peak amplitude), peak amplitude (point with the highest energy within the spectrum), minimum/maximum frequency and bandwidth (difference between the USV's minimal and maximal sound frequency in kHz of each detected USV). As temporal parameters, mean USV duration/call length and the rate of USVs emitted were evaluated. Total calling time was calculated for each subject by adding individual call lengths. Average total calling time was calculated for each strain under different contexts.

2.6 Statistical analysis

Data are expressed as group mean±SE. Parametric tests with or without repeated measures were used for context-specific and strain-specific differences respectively. The level of significance was defined as $p \leq 0.05$.

3. Results

The 50 kHz USVs that were emitted consisted of flat, frequency modulated and frequency step subtypes based on their sonographic patterns. Flat (F) were USVs which resembled a straight line; i.e. with no more than one change in frequency direction not exceeding 5 kHz. Frequency Modulated (FM) USVs were wavy with more than 5 kHz frequency difference within a wave. They had a minimum of two waves. Frequency Step (FS) had a jump in frequency of more than 5 kHz (FS). These were complex USVs with steps and sudden jumps in frequency. All the 50 kHz USVs had frequencies above 33 kHz and were labeled for further analysis, though short USVs of lower frequency (between 28 and 32 kHz) were also observed in both strains across contexts, more so in WKYs than in Wistars.

On the spectrogram (figure 1), flat USVs emitted by Wistars appeared either as straight lines that faced up or down, as waves or as inverted 'U's, 'V's and 'M's, the change in frequency direction in these not exceeding 5 kHz within an USV. Flat USVs terminated either diagonally or as a small hump. Short, 2–5 ms long USVs emitted in succession or successive discontinuous inverted U-shaped USVs were also observed.

After emitting 5–15 flat USVs, a switch to FS or FM USVs was observed, before returning again to flat USVs. Among FMs, USVs with increased frequency modulation were also observed. FS USVs consisted of flats with a step-down or step-up, or a combination of step-ups and step-downs, the difference in frequency being greater than 5 kHz (figure 1, upper panel). In WKYs, flat USVs comprised upward/downward flats, wavy flats, successive inverted U-shaped flats, upward flats with a step-up and step-down or a step-up component that occurred 5 ms after a flat USV, all within a variation of 5 kHz. Long wavy FM USVs of about 270 ms were also observed (figure 1, lower panel). In 75% of Wistars and 57% of WKYs, irrespective of context, flat USVs were emitted at the start; that is on introduction into the testing arena, and towards the end of the recording.

3.1 Cage test USVs

Fourteen of the Wistars and fifteen of the WKYs tested in a cage with fresh bedding emitted 50 kHz USVs (figure 2). Of these, 92% Wistars and 57% WKYs emitted all the three USV subtypes, while the remaining emitted only flat USVs throughout the testing period. FM and FS USVs were emitted either in quick succession, followed again by a series of 10–15 flat USVs or were emitted alternatively with flat USVs. If one subtype was emitted first, this subtype continued to be emitted before another type was emitted. For instance, the FM or FS USVs were observed after 39±10 s in Wistars, while in WKYs, they were observed only after 98±34 s. The majority (92% Wistars and 71% WKYs) emitted flat USVs first, with the average latency (6.55±1.94 s) to the first flat USV in Wistars being significantly shorter ($p<0.01$) than in WKYs (46.62±10.91 s).

Wistars emitted significantly more number of 50 kHz USVs ($p<0.05$) averaging at 30.33±5.76 in comparison to WKYs (14.35±4.08). As most of these USVs were flat USVs, Wistars emitted significantly more ($p<0.05$) flat USVs than WKYs. Very few FM and FS USVs were emitted by both strains (figure 2). Mean ambulatory activity in the cage as also rearing frequency was comparable between strains.

3.2 EPM USVs

The latency to emit a flat USV in the EPM was not significantly ($p>0.05$) longer in WKYs (36.10±13.25 s) than in Wistars (20.6±8.38 s). USVs emitted by Wistars were significantly lower ($p<0.001$) than in the cage, while WKYs showed no significant difference in USV emission when compared to the cage. The majority of 50 kHz USVs were
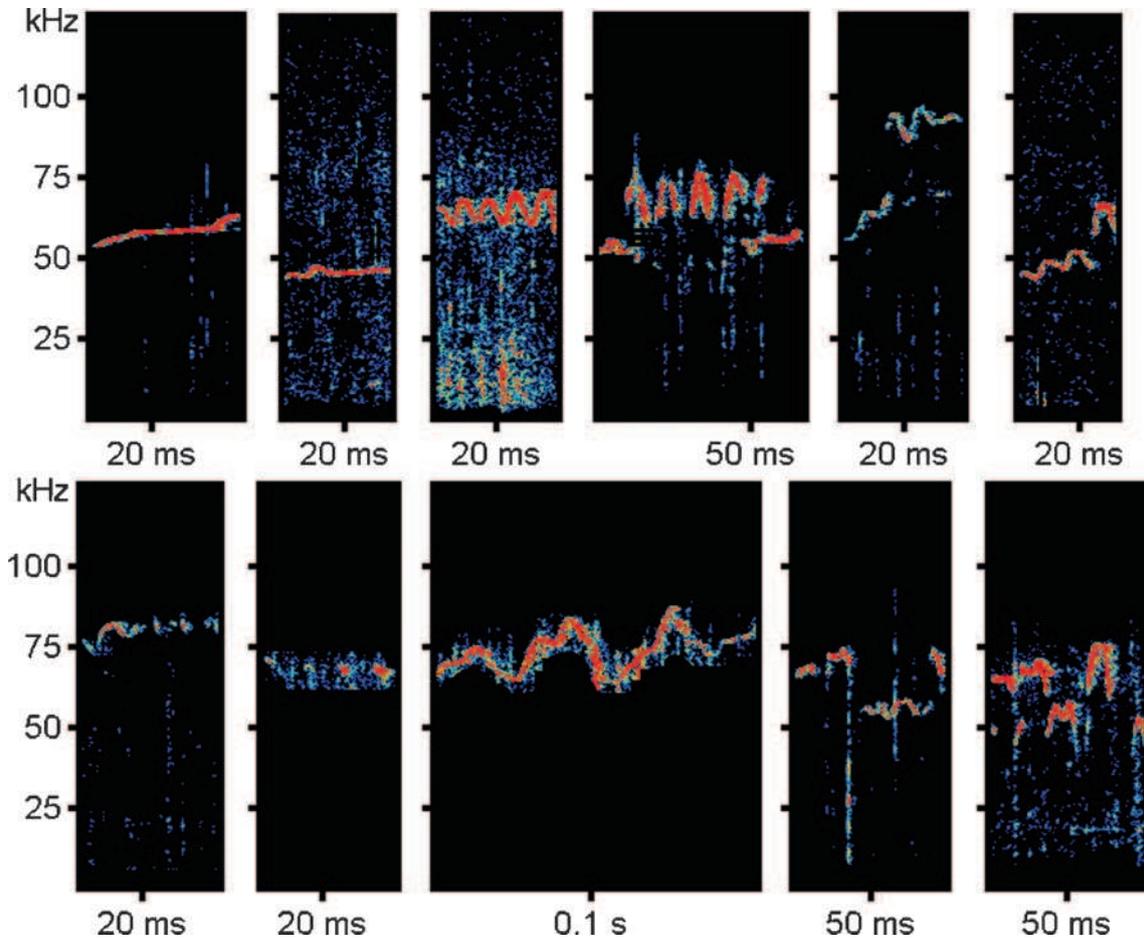
Figure 1. 50 kHz USV types and subtypes. The upper panel shows representative USVs of each category; from the left, two flat, two frequency-modulated (FM) and two frequency step (FS) USVs emitted by Wistars. Wistar FMs show continuity until an USV is complete. FS USVs have fewer step-up and step-down combinations in a single USV. The lower panel shows representative USVs of WKYs; from the left, two flat (F), two frequency-modulated (FM) and two frequency step (FS) USVs. Short successive inverted U's and short successive downward flats were emitted often. WKY FS USVs have a higher combination of step-ups and step-downs in a single USV.

again of the flat category (in Wistars: 92.06%; in WKYs: 95.32%) with significant inter-strain group mean differences ($p<0.05$). No FM USVs were observed in Wistars and very few were observed in WKYs. FS USVs were also very few in both strains (figure 2).

Behavioural measures such as latency to enter open arm, open arm entries, open arm time and centre time were comparable between strains (figure 3 a,b,c,d), as also distance moved, rearing frequency and dipping frequency (figure 3 e,f,g,h). USV number was not significantly correlated with open arm time, in both Wistars (r=0.213, $p>0.05$), as well as in WKYs (r=−0.216, $p>0.05$).

On subsequent exposure to the EPM, there is no difference in the USVs emitted in WKYs, though Wistar USVs continued to be significantly lower ($p<0.05$) than in the cage (figure 2). Latency to emit USVs (flat) was significantly longer ($p<0.05$) in Wistars with 38.88±9.94 s, while in WKYs it was just 5.36±3.78 s. Very few FM and FS USVs were emitted by both Wistars and WKYs. The inter-strain difference ($p<0.05$) in USV number that was observed in EPM-1 was no more evident in EPM-2. On second exposure, USV frequency was not significantly correlated with open arm time, in both Wistars (r=−0.242, $p>0.05$), and WKYs (r=0.216, $p>0.05$).

3.3 *Temporal characteristics of USVs*

Wistars emitted more USVs during the first and second minutes in the cage context, with a gradual decrease subsequently (figure 4a). Wistars that did not emit USVs in the first minute emitted very few USVs throughout the testing
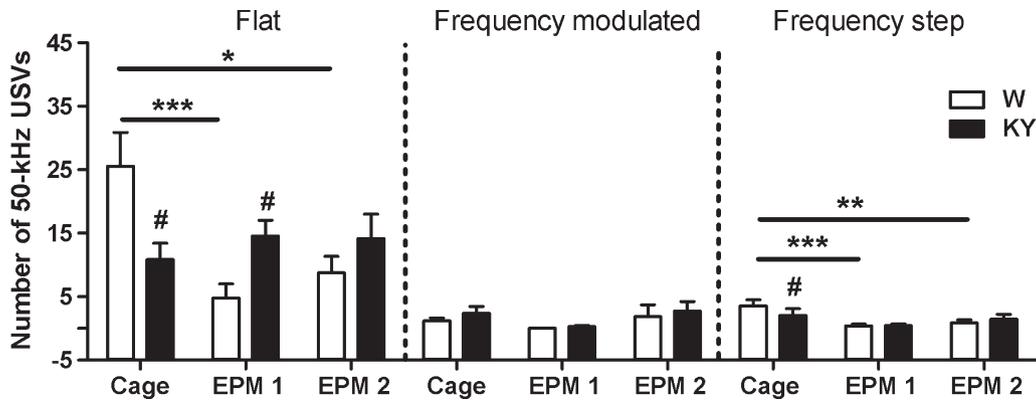
Figure 2. Context-specific differences in frequencies of 50 kHz USV subtypes: Flat, frequency-modulated and frequency step USVs were compared across cage and EPM. Wistars: Significantly more flat 50 kHz USVs were emitted in cage compared to EPM-1 ($p<0.001$) and EPM-2 ($p<0.05$). Frequency-modulated USVs were not emitted in EPM-1. Frequency step USVs emitted in cage vs. EPM-1 ($p<0.01$) and EPM-2 ($p<0.01$) were significantly reduced. WKY: No difference was observed in the number of all 50 kHz USV types in cage when compared to EPM-1 and EPM-2.

period. Individuals that demonstrated a higher USV rate, started emitting USVs within the first few seconds of introduction into the testing arena (either cage or EPM), whereas low callers start emitting USVs only after about 20 s or so. 40% of WKYs did not emit any USVs in the first minute. WKYs mostly emitted USVs in second and third minutes of the recording (figure 4b).

When we consider total calling time in different contexts (figure 4c), Wistars emitted USVs in 0.69±0.23 s in the cage. The time spent calling went down significantly ($p<0.05$) in EPM-1 and EPM-2 ($p<0.01$). WKYs emitted USVs for a total of 0.82±0.26 s in the cage, which reduced to 0.14 ±0.03 s in the EPM, the difference being significant ($p<0.01$). On re-exposure, WKYs emitted USVs for 0.40 ±0.15 s. There is a significant difference between strains in calling time in EPM-2 ($p<0.05$).

3.4 *Acoustic characteristics of USVs*

In order to detect differences in USV emissions that were strain-specific or context-specific, spectral and temporal characteristics of 682 individually marked USVs were analyzed in more detail. In the cage, WKY USVs were found to be significantly longer ($p<0.001$) than Wistar USVs. Signal strength (peak amplitude) of WKY USVs was significantly stronger ($p<0.01$). USV bandwidth, which indicates extent of modulation in USVs, peak frequency, that is strongest frequency component at highest amplitude, minimum and maximum frequencies were comparable between strains.

In the EPM, on first exposure, USV length or duration and USV bandwidth were comparable between strains. USV peak amplitude was weaker in WKY USVs when compared

to Wistar USVs ($p<0.001$). Peak frequency ($p<0.001$), minimum frequency $p<0.001$ and maximum frequency ($p<0.001$) were significantly lower in WKY USVs than in Wistar USVs, despite the fact that some WKY USVs had peak frequencies up to 123 kHz (minimum frequencies of 107.4 kHz and maximum frequencies of 124 kHz).

In EPM-2, USV length or duration was different between strains, with WKY USVs being significantly longer ($p<0.05$). Peak amplitude was stronger in WKY USVs ($p<0.001$). USV bandwidth was significantly lower in WKY USVs ($p<0.05$).

When compared to cage, EPM exposure induced a significant decrease in USV length or duration in both strains. On re-exposure, USV length goes back to the length in cage in both Wistars and WKYs. WKY USVs which have higher peak amplitudes than Wistar USVs demonstrate a significant decrease in EPM-1 and EPM-2 when compared to cage, though peak amplitude is significantly increased on re-exposure to the EPM. Peak amplitude of Wistar USVs, on the other hand, was found to significantly decrease on re-exposure. Bandwidth of WKY USVs increases significantly in EPM-1. For all the data (range, mean±SE) and significant differences in spectral characteristics between strains under the same context and within the same strain between contexts, see table 1.

4. Discussion

Results indicate the existence of inter-strain differences in certain features of 50 kHz USVs as has also been shown in Long Evans (Panksepp and Burgdorf 2000; Wright *et al.* 2010) and Sprague Dawley (Taracha *et al.* 2012; Willey and Spear 2012). WKYs emitted long 50 kHz USVs, not
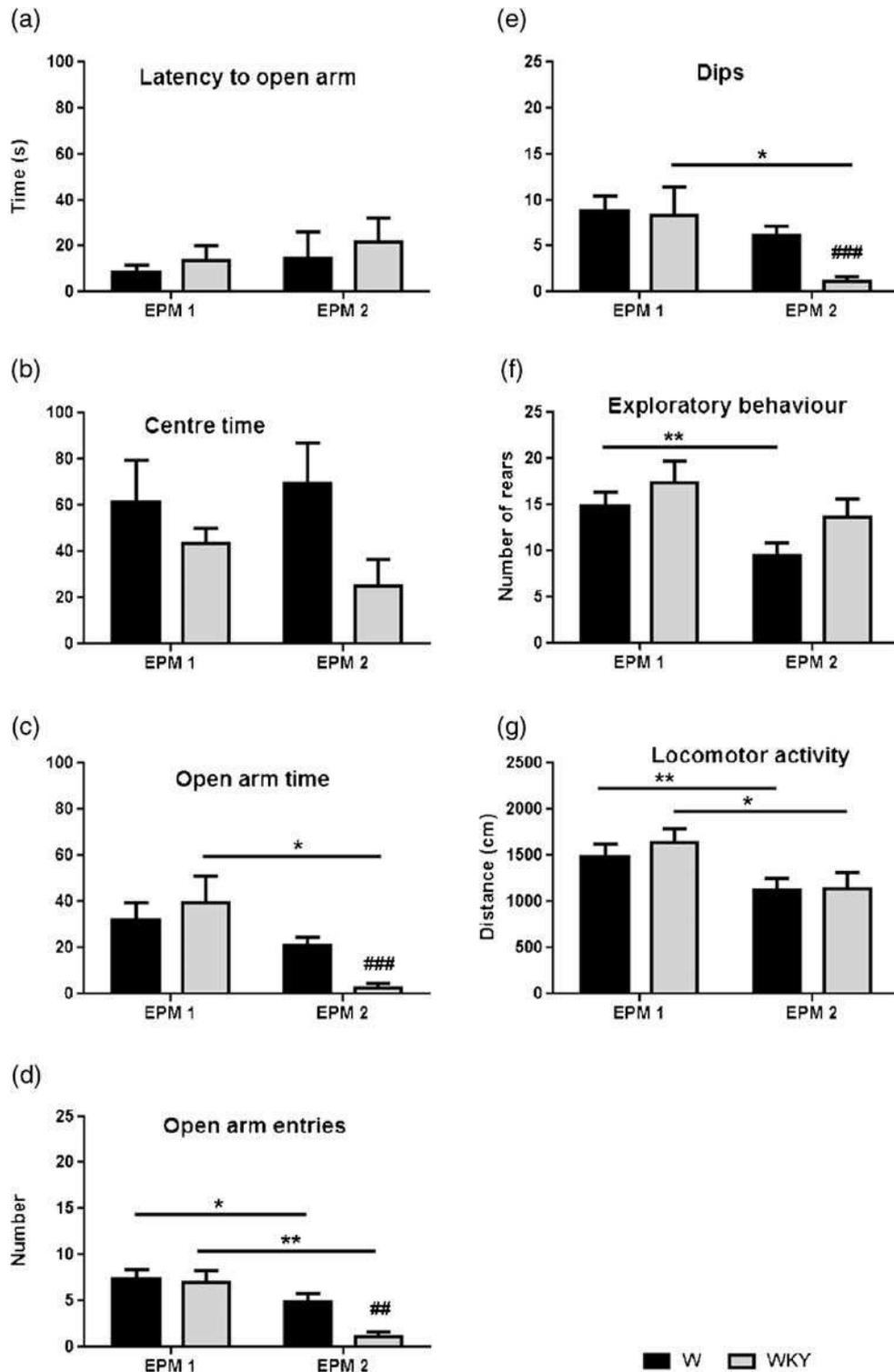
Figure 3. EPM behavioural measures in Wistars and WKYs: None of the parameters measured in EPM-1 were significantly different between strains. Rearing was defined as 'raising of the forelimbs in the air or on a wall'. Dipping was defined as 'stretching of the head from the closed arm or head dipping towards the floor from open arm or centre'. In EPM-2, open arm time (**c**), open arm entries (**d**) and dipping frequency (**e**) were very significantly different between strains. $*p<0.05$; $**p<0.01$ strain-specific differences between contexts; $^{\#\#}p<0.01$ $^{\#\#\#}p<0.001$ context-specific strain differences.
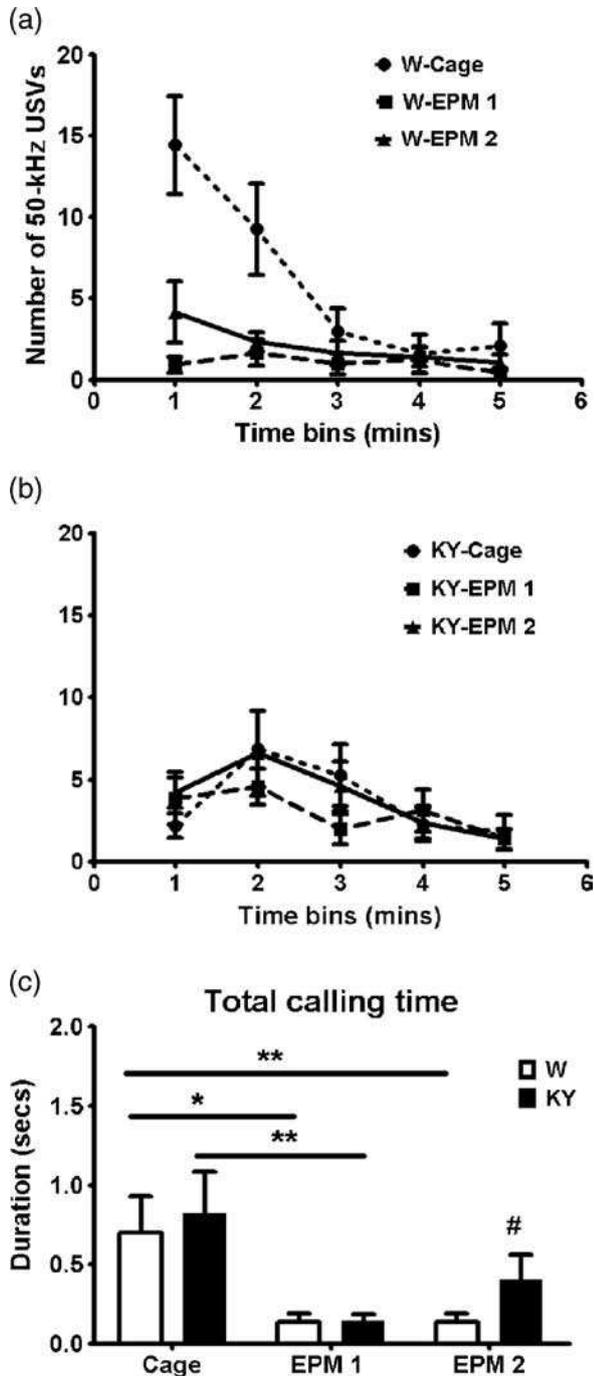
Figure 4. Temporal characteristics of USVs emitted by Wistars and WKYs in different contexts. (**a**) 1 min bins of USVs across different contexts in Wistars and (**b**) WKYs. (**c**) Time spent calling or total calling time in seconds (secs) across contexts and between strains. There is a significant decrease in calling time in EPM-1 in both Wistars ($p<0.05$) and WKYs ($p<0.01$) when compared to cage. Re-exposure to the EPM has no effect on Wistars, although there is an increase in WKY calling time, which is significant when compared to Wistars ($p<0.05$).

irrespective of the context tested in, while Wistars demonstrated a decrease in USV emission in the EPM that persisted even on repeated exposure, indicating that EPM-induced anxiety levels attenuated 50 kHz USVs in Wistars, but had no effect on WKY USVs. This is the first study of adult USV emission and behaviour in different contexts in the WKY in comparison to its parent Wistar strain, the only other comparative study being in WKY pup USVs emitted on maternal separation (Braw *et al.* 2008).

4.1 *Cage test USVs*

Besides indicating emotional/motivational states and reward valence (Knuston *et al.* 1999), USVs are also said to index general arousal, seeking behaviour (Schwarting *et al.* 2007, Burgdorf *et al.* 2011), and serve as social signals (Wöhr and Schwarting 2012), although they have also been observed in non-social contexts (Wöhr *et al.* 2008). The cage serves as a social context; the reduced USV emissions shown here by WKYs indicates their inherent behavioural inhibition, as typified by a reserved response or inactivity in the face of novel social and non-social situations (Servatius *et al.* 2008). The differences in USVs observed in both WKYs and Wistars are stable, and could reflect underlying 'trait'-like characteristics that have been shown to be consistent (Borta *et al.* 2006; Schwarting *et al.* 2007; Taracha *et al.* 2012). For instance calling rate is the same when tested over days in different paradigms (this study – results not shown; Mällo *et al.* 2007, Wöhr *et al.* 2008). Whether individual rats differ in terms of the specific USV types that they preferentially emit, or whether a so-called USV 'profile' exists, needs to be explored further.

Flat USVs, the major USV type observed here, are said to constitute contact calls as they occur at higher rates during non-positive affective social interactions. They should ideally be stereotyped, repetitive and cover a broad bandwidth, or have rapid changes in frequency (Sales 2010), as was also observed here. These are capable of influencing a conspecific's behaviour (Blanchard *et al.* 1991), as playback elicits approach (Sadananda *et al.* 2008) and communicative responses (Wöhr and Schwarting 2007). The flat USVs emitted at the beginning of the recording, which is on immediate separation from cage mates, become fewer later on, suggesting that flat USVs are separation-induced and indeed have a communicative value. The motivation to emit an USV is strongest on separation from a cage mate and/or on initial exploration of the testing arena. After the second minute, small 2 ms UVSs were observed. When emission of neither FM nor FS USVs seemed to establish contact with cage-mates, rats emitted flats again (Wright *et al.* 2010). The low number of FM and FS USVs is not unexpected given that these are emitted in greater number on tactile stimulation or tickling (Schwarting *et al.* 2007) and in anticipation of reward (Thompson *et al.* 2006).

observed in Wistars. USV rates also differed based on the context with WKY showing comparable USV emission

Table 1. Mean spectral characteristics of 50 kHz USVs emitted by Wistars and WKYs under different contexts

	Cage		EPM-1		EPM-2	
	W (n=143)	WKY (n=144)	W (n=59)	WKY (n=101)	W (n=110)	WKY (n=125)
Call length (ms)	14.66±0.87 (2.5 to 51.7)	22.89±1.90[###] (3 to 158.7)	11.6±1.12[$] (3 to 62.4)	10.13±0.50[$$$] (2.5 to 76.2)	14.73±1.28 (2.5 to 69.1)	19.45±1.71[#] ,[***] (2 to 86.5)
Peak frequency (kHz)	55.63±1.37 (34.6 to 103)	58.12±4.84 (34.1 to 93.2)	63.55±2.05[$$] (35.1 to 99.1)	52.57±5.82[###],[$$] (34.1 to 123)	56.76±1.41[**] (34.6 to 106.4)	57.47±1.14[**] (34.1 to 79.5)
Peak amplitude (dB)	−60.23±0.73 (−79.33 to −36.89)	−57.39±4.78[##] (−76.41 to −36.67)	−61.20±1.16 (−75.25 to −33.88)	−65.40±6.74[###],[$$$] (−77.75 to −32.96)	−66.40±0.50[***] (−76.41 to −45.46)	−63.56±0.62[###],[*] (−79.33 to −40.68)
Bandwidth (kHz)	5.42±0.56 (0.9 to 45.8)	4.90±0.40 (1.4 to 42.4)	7.31±1.21 (0.9 to 43.9)	7.70±0.67[$$] (0.9 to 46.8)	9.16±0.85 (1.4 to 46.3)	6.8±0.63[#] (1.4 to 43.4)
Min. frequency (kHz)	53.75±1.35 (33.2 to 102)	55.96±4.66 (33.2 to 92.7)	60.86±2.12[$$] (33.2 to 98.6)	49.66±5.48[###],[$$$] (33.2 to 107.4)	52.62±1.28[***] (34.1 to 95.7)	54.9±1.09[**] (33.6 to 78.1)
Max. frequency (kHz)	59.23±1.41 (36.6 to 115.2)	60.91±5.07 (38.5 to 96.1)	68.22±1.98[$$$] (38 to 103.5)	57.41±6.17[###],[$] (35.6 to 124)	61.84±1.51[*] (36.6 to 110.3)	61.78±1.15[*] (36.6 to 85.4)

Range is given in brackets; # indicates significant strain differences in the same context; $ indicates context-based (cage vs. EPM-1) differences within a strain; * indicates context-based (EPM-1 vs. EPM-2) differences within a strain.

Cage vs. EPM-1: Wistars – USV length or duration ($p<0.05$) was significantly reduced while peak frequency ($p<0.01$), min. frequency ($p<0.01$), max. frequency ($p<0.001$) were significantly increased. In WKYs besides USV length or duration ($p<0.001$), peak frequency ($p<0.01$), amplitude ($p<0.001$), min. frequency ($p<0.001$) and max. frequency ($p<0.05$) were significantly reduced. Bandwidth ($p<0.01$) increased significantly.

EPM-1 vs. EPM-2: Wistars – Peak frequency ($p<0.01$), amplitude ($p<0.001$), min. frequency ($p<0.001$), max. frequency ($p<0.05$) varied significantly. WKYs – USV length or duration ($p<0.001$), peak frequency ($p<0.01$), amplitude ($p<0.05$), min. frequency ($p<0.01$), max. frequency ($p<0.05$) were significantly increased.

4.2 EPM USVs

The significant reduction of USVs in the EPM when compared to the cage reflects avoidance behaviour as the EPM is an un-conditioned test of anxiety and measures the conflict that exists between the animal's exploratory drive and its innate fear of open spaces (Schneider *et al.* 2011). A reduction in 50 kHz USVs is observed in frustrating, non-rewarding situations (Burgdorf *et al.* 2007). During a second trial, WKYs spent more time calling, but demonstrated no difference between USV numbers emitted in both exposures. USVs were found to increase in Wistars on repeated exposure, although not significantly. That the maze is less aversive on re-exposure (Wehrmeister *et al.* 2010, Rao and Sadananda 2011) or that prior exposure caused a shift in the animal's emotional state (Cohen *et al.* 2008) could underlie the increase in USVs in Wistars. Alternately, it could be that repeated testing (cage, EPM-1, EPM-2) led to the significant reduction in USV emission at least in Wistars, as has been shown, with trained rats emitting fewer USVs than naïve rats (Wöhr *et al.* 2008), although other studies showed no such correlation (Schwarting *et al.* 2007).

In WKYs, on the other hand, repeated testing does not have the same effect as has been shown also in other behavioural paradigms, such as the emergence test (Paré *et al.* 2001). Similarly, no increase in USV emission was observed in WKY pups on repeated separation from the dam (Braw *et al.* 2008), when compared to Wistar pups. On first separation in the cage test here, WKYs emitted fewer USVs than Wistars, with EPM exposure and re-exposure inducing no change in USV number. Whether the similar USV frequency observed in the three diverse contexts, or rather, irrespective of change of context, reflects the psychopathology of the strain needs to be examined further. Stable strain differences in behaviour and underlying neurochemistry (Langen and Dost 2011) make such an assumption plausible.

4.3 Acoustic-temporal characteristics of USVs

Parameters most frequently used for measuring vocal behaviour are USV number, length and amplitude. If number is considered as the most salient feature, it was comparable across contexts in WKYs, while in Wistars it was greatest in the cage and significantly reduced in the EPM. If mean USV length is considered, WKY USVs were much longer than Wistar USVs in the cage. In EPM-1, lengths were found to be reduced in both strains. On re-exposure to the EPM, USV length increased, again suggesting that the EPM is less aversive during the second exposure.

From the spectral analysis we detected acoustic characteristics that differ in USVs emitted in the cage test and

EPM. The signal strength in terms of peak amplitude was stronger in WKY USVs under all contexts. This decreased in EPM-1 and increased on re-exposure in WKY USVs, while it remained unchanged in Wistars irrespective of context. Bandwidth did not appear to be affected by context and was not different between strains, while peak frequency, which is the strongest frequency component at highest amplitude, was lower in WKY USVs than in Wistar USVs when exposed to the EPM. While certain spectral characteristics may be genetically based, others are modulated by external cues. For instance, features such as USV frequency and amplitude have been shown to be primarily genotypic as embryo-transfer procedures (Wöhr *et al.* 2008) have proved, while USV number and bandwidth are based on extraneous, contextual factors, as has been shown in isolated rat pups (Brudzynski *et al.* 1999).

Here, 50 kHz USVs emitted by the putatively endogenous depressive WKY strain in different contexts have been classified; their acoustic, temporal and spectral characteristics quantified in comparison to the parent Wistar strain. The significantly reduced USV emission of WKYs, when compared to Wistars, remains stable irrespective of the context exposed to. The EPM exposure induces an anxiety-like profile in behavioural measures, with no effect on USV number, though there was a significant reduction in call length and amplitude in WKY USVs. Repeated exposure to EPM induced no change in USV emissions, though call length and amplitude increased, the former to levels emitted in the cage context. While assessment of the effect of contextual factors on USV emission could be used to unravel the significance of social and proximal cues, studies using different strains do throw light on whether USVs emitted reflect inherent traits and thereby constitute non-invasive read-outs.

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