Acta Neuropsychiatrica

cambridge.org/neu

Original Article

Cite this article: Kai LM, Iglesias LP, Kõiv K, Harro J, and Wegener G. (2025) Ultrasonic vocalisations in the Flinders Sensitive Line rat, a genetic animal model of depression. *Acta Neuropsychiatrica*. **37**(e3), 1–8. doi: 10.1017/neu.2024.61

Received: 29 May 2024
Revised: 7 November 2024
Accepted: 3 December 2024

Keywords:
Rats; anhedonia; vocalisation; depression; reward

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Ultrasonic vocalisations in the Flinders Sensitive Line rat, a genetic animal model of depression

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Abstract

Objective: Ultrasonic vocalisations (USVs) emitted by rats may reflect affective states. Specifically, 50 kHz calls emitted during juvenile playing are associated with positive affect. Given that depression is characterised by profound alterations in this domain, we proposed that USV calls may configure a suitable tool for assessing depressive-like states. Utilising the Flinders Sensitive Line (FSL), a well-established animal model of depression, we assessed USV calls emitted by rats during tickling, a procedure based on juvenile rats' rough-and-tumble play. *Methods:* Juvenile FSL rats and their control counterparts, the Flinders Resistant Line (FRL) and Sprague Dawley, were submitted to tickling sessions to imitate rats playing behaviour. The rats were tickled daily for 6 weeks starting at PND21. Tickling sessions were recorded for further acoustic analysis of 50 kHz calls. *Results:* Tickling increased 50 kHz calls in all the strains. FSL rats emitted more calls than control strains and exhibited a higher number of flat-trill combination calls. Conclusion: Tickling is a robust method for inducing 50 kHz USV calls. Analysing USV calls emitted during tickling configurates a suitable method for studying affective states relevant to depression. FSL rats did not present anhedonia but rather higher reward sensitivity, which may underlie their stress vulnerability.

Significant outcomes

- Tickling induced 50 kHz USV calls in FSL and SD young rats.
- Tickling induced a higher 50 kHz USV response in FSL than SD or FRL.
- USV pattern can be used to infer emotional states in rodents.
- FSL does not seem to be a suitable model for studying anhedonia.
- FSL may serve as a model of non-adaptive reward sensitivity.

Limitations

- Animals were single-housed to increase playing behaviour during tickling sessions.

Introduction

Depression is a severe and life-threatening disease with extensive personal and societal costs (Whiteford *et al*., Whiteford *et al*., 2013). Despite the recent advancements (Krystal *et al*., 2024), current pharmacological treatments are unsatisfactory as most present a delayed onset, limited efficacy and poor long-term symptom control (Dwyer & Duman, 2013, Gigliucci *et al*., 2013). Thus, there is an urgent need to refine the preclinical methods used to discover putative antidepressants (Cryan & Lucki, 2002, Neumann *et al*., 2011, Gururajan *et al*., 2019). The forced swim test is a predictive tool to assess antidepressant-like behaviour (Porsolt *et al*., 1977a; Porsolt *et al*., 1977b; Porsolt *et al*., 1978, Cryan & Lucki, 2002, Cryan & Slattery, 2007, Slattery *et al*., Slattery & Cryan, 2012). Despite being highly popular due to its feasibility and reproducibility, its use, interpretation and conceptualisation are criticised.

Rats emit ultrasonic vocalisations (USVs), inaudible to the human ear. These reflect the affective state of the rats (Knutson *et al*., 1999). For instance, when rats have their tails pinched or are exposed to other aversive stimuli, they elicit 22 kHz vocalisations associated with negative affect (Panksepp, 1999, Knutson *et al*., 2002). Similarly, maternal separation induces calls of 40 or 60 kHz (Boulanger-Bertolus *et al*., 2017; Kaidbey *et al*., 2019). Alternatively, administration of amphetamine either systemically or directly in the nucleus accumbens, a vital structure of the reward system, was linked to 50 kHz vocalisations (Burgdorf *et al.,* 2001; Burgdorf & Panksepp, 2001). These vocalisations are also spontaneously elicited when juvenile rats play with each other, suggesting its association with positive affect (Panksepp, 1999, Knutson *et al.*, 2002). The experimenter can imitate the juvenile rats' rough-and-tumble play through a tickling-like stimulation, increasing 50 kHz vocalisations. The number of calls elicited can be used to infer depressive and anxiety-like behaviour (Burgdorf *et al.,* 2001; Mallo *et al.,* 2007).

The Flinders Sensitive Line (FSL) is an inbred line widely validated as a model of depression (Overstreet, 1992, Overstreet *et al.*, 2013). Studies on face validity demonstrate: (I) reduced sucrose intake following stress and increased immobility in the forced swim test, suggesting a depressive-like phenotype (Wegener G., 2012); (II) elevated REM sleep and impairments in the novel object recognition task, modelling disturbed sleep and cognitive deficits related to depression (Wegener *et al.,* 2012). Studies on construct validity in this model show serotonergic, cholinergic, neurotrophic and morphological abnormalities, which have been implicated in depression in humans (Overstreet & Wegener, 2013; Strenn *et al.*, 2015; Ardalan *et al.*, 2017; Kirkedal *et al.*, 2019; Tillmann & Wegener, 2019, Treccani *et al.*, 2019; Abildgaard *et al.*, 2021; Arjmand *et al.*, 2023; Domingos *et al.*, 2024). Emphasising its predictive validity, the FSL rat shows decreased immobility in the forced swim test when treated chronically and acutely with drugs that have antidepressant effects in humans (du Jardin *et al.*, 2016; Marchetti *et al.*, 2020). Therefore, the present study examined the emission of USVs during tickling stimulation in the FSL rat compared to the Flinders Resistant Line (FRL) and outbred Sprague Dawley (SD) rats – both commonly used controls for the FSL rat.

The aims of the study were to i) characterise and ii) investigate the applicability of USVs as a screening method for depressive-like behaviour in a rodent model of depression. It was hypothesised that the depressive-like phenotype of the FSL rat would be reflected in the ultrasonic response to tickling stimulation.

Methods

Animals

Juvenile 22-day-old male FSL (n = 27), FRL (n = 24) and SD (n = 23) rats were obtained from the in-house breeding colony at the Translational Neuropsychiatry Unit, Aarhus University (Denmark). Animals were housed at 22 ± 2°C and kept at a 12-hour light/dark cycle (lights on at 07:00) with access to food and water *ad libitum*. The rats had access to a hide, nesting material and a wooden stick throughout the experiment. All animal procedures were approved by the Danish National Committee for Ethics in Animal Experimentation (permission ID: 2012-15-2934-00254). Baseline characteristics of the animals are given in Table 1.

Experimental design

On postnatal day 21, the rats were weaned from their mothers. All rats were single-housed during the entire experimental period to increase play behaviour during tickling sessions (Panksepp, 1981; Panksepp *et al.*, 1984). The rats were randomly assigned to the experimental groups (tickling/light-touch). All experimental procedures were carried out in the rat's light cycle.

Tickling

The rats were tickled daily for 6 weeks starting from postnatal day 21. A tickling session consisted of 15 s acclimatisation to the tickling cage followed by 15 s hand-play with the experimenter, touching the nape of the neck and abdomen, then 15 s of no stimulation followed by 15 s tickling. During the last three seconds of each bout of tickling, attempts were made to pin down the rat and vigorously tickle the abdomen. This method has been described elsewhere (Knutson *et al.*, 1998; Mallo *et al.*, 2007). A dorsal light-touch group was used as a control group for the tickled rats. As previously described, light touch has been used as a control for tickling, as it is a discernible stimulation but presumably with less reward value (Burgdorf *et al.,* 2001; Burgdorf & Panksepp, 2001; Yamamuro *et al.,* 2013). Acoustic foam was used to isolate the Plexiglas container where the tickling took place (Brudzynski & Pniak, 2002). The same experimenter conducted all the tickling sessions and was blinded to the strain of the rats.

Acoustic data acquisition, analysis and classification

The tickling sessions were recorded using Avisoft-RECORDER USGH (v.4.2 Avisoft Bioacoustics, Berlin, Germany). A condenser microphone CM16/CMPA (from AvisoftBioacoustics) was secured 20 cm from the cage floor, and its signal was fed to the Avisoft UltraSoundGate 416H with a sampling rate of 750 kHz. Acoustical analysis was performed with the Avisoft SASlab Pro (v.5.2 Avisoft bioacoustics, Berlin, Germany), and spectograms were generated with a fast Fourier transformation (FFT)-length of 256 points and an overlap of 75% (FlatTop window, 100% frame size). A semi-automatic recording of call parameters was used for the quantitative analysis of the 2-minute tickling or light-touch sessions. In accordance with Reno *et al.* (2013), calls with a frequency between 30–90 kHz were considered 50 kHz calls and between 20 and 30 kHz were considered 22 kHz calls (Reno *et al.*, 2013). Furthermore, calls were regarded as individual vocalisations when separated by at least 20 ms. The minimum call length was set at 5 ms and sounds shorter than this were considered noise. For the 50 kHz calls, the differences in morphology and call subtypes were based on the classification by Wright et al. (Wright *et al.*, 2010). This classification consists of 14 call categories differently modulate by diverse stimuli, environmental conditions and inter-individual differences, ultimately allowing a more refined analysis of the affective state (Wright *et al.*, 2010). The qualitative evaluation of the calls was carried out on the first 15 s stimulations of experimental day 23, as this was the day with the largest quantity of calls. The categorisation was made manually based on the predefined frequency patterns described by Wright et al.

Data analysis and statistics

All statistics were conducted using SPSS version 22 and GraphPad Prism 10 for Windows (GraphPad Software, San Diego, CA). The statistical analysis of differences between periods of tickling versus periods without tickling and among ticking sessions was done with repeated-measures one-way analysis of variance (ANOVA) with planned post hoc pairwise comparisons using Geisser-Greenhouse correction for sphericity and Bonferroni for multiple comparisons. The statistical analysis of differences between the tickling and the light-touch groups or strain differences for call categories was done using a two-way ANOVA with post hoc Bonferroni for multiple comparisons. Spearman's correlation coefficient was used to determine the inter-individual stability of calls between days.

https://doi.org/10.1017/neu.2024.61 Published online by Cambridge University Press

Table 1. Group characteristics. Group distributions of age and weight at the beginning of the experiment. Values are expressed as means ± SD. FSL = Flinders Sensitive Line rat, FRL = Flinders Resistant Line rat, SD = Sprague Dawley

	FSL		FRL		SD	
Group	Tickled	Light-touch	Tickled	Light-touch	Tickled	Light-touch
Group size	16	8	16	11	16	7
Age (days)	21.6 (±1.1)	21.9 (±1.4)	21.5 (±0.8)	21.9 (±0.8)	23 (±0.8)	22.8 (±0.9)
Weight (day 3)	54.6 (±7)	53.2 (±5.6)	61.8 (±9)	56.6 (±13)	70.3 (±18)	72 (±19)

The qualitative differences in calls between strains were analysed by a one-way ANOVA, followed by a Tukey post hoc test. A two-tailed p-value of less than 5% was considered significant for all tests.

Results

Ultrasonic vocalisations

More than 71.000 calls were counted, and more than 2.100 were classified according to Wright et al., categories (Wright *et al.*, 2010). As 22 kHz calls accounted for less than 2.9 % of calls on day 1 and 0.6 % on day 6, these were not further analysed. Also, 40 of the 74 rats produced calls which could not be classified as 50 kHz as their frequency was below 30 kHz. They did not have the appearance of 22 kHz either, as they were shorter (<300 ms) or presented frequency modulations. This type of call may be reminiscent of infants' vocalisations. They were primarily present in the first days of recording, after which they decreased. On experimental day 13, only three rats produced these calls, and they were not further analysed. Finally, in the qualitative evaluation of the call profiles, 26% of the calls could not be classified and went into a miscellaneous group.

Tickling increases the production of 50 kHz calls

The number of calls was significantly different among strains and stimulation type on both days, 1 [Strain: F (2, 66) = 7.358, $p = 0.0013$; Stimulation: F (1, 66) = 47.75; $p < 0.0001$; Interaction: F (2, 66) = 5.334, $p = 0.0071$] and 23 [Strain: F (2, 66) = 11.07, $p < 0.0001$; Stimulation: F (1, 66) = 132.5, $p < 0.0001$; Interaction: F (2, 66) = 8.123, $p = 0.0007$]. The number of calls was higher in tickled animals. This difference was not statistically significant for the FRL rats on day 1 (Fig. 1). Moreover, while no differences among strains were noticed in the light-touch group, tickled FSL emitted more calls than FRL and SD on both days.

Furthermore, in the tickled group (Fig. 2), there was a significant difference in the number of calls emitted between periods of stimulation and no-stimulation [Strain: F (2, 45) = 0.8199, $p = 0.4427$; Time Interval: F (2.707, 121.8) = 17.03, $p < 0.0001$; Interaction: F (14, 315) = 0.6060, $p = 0.8599$].

The FSL rat produces more calls than their controls during tickling

Quantitative analysis of calls during tickling revealed a significant effect of strain, day and interaction on the number of calls produced [Strain: F (1.991, 179.2) = 127.9, $p < 0.0001$; Day: F (5, 90) = 13.17, $p < 0.0001$; Interaction: F (10, 180) = 3.014, $p = 0.0015$]. Post hoc analysis showed that FSL emitted more calls than FRL rats. Moreover, apart from day 1, FSL also produced more calls than SD, and on days 13 and 23, FRL emitted more calls than SD (Fig. 3).

Furthermore, a correlation study was performed to evaluate whether the quantity of calls was temporally stable. FSL rats did not show a correlation between days; however, SD (from day 6) and FRL (from day 13) did (see Table 2).

Qualitative call profiles among strains

Lastly, 57 and 2074 calls were classified in the light-touch and tickled groups, respectively. The qualitative analysis is graphically presented in Fig. 4. The percentage of calls of each type can be found in the S1 table.

The qualitative analysis of the calls for each strain in the tickled group is graphically presented in Fig. 5A-C. The percentage of each call type can be found in Table S2. No differences in the relative number of calls were found among strains for trill [Figure 5D: F (2, 45) = 0.3944, $p = 0.6764$], flat [Figure 5E: F (2, 45) = 1.308, $p = 0.2804$], multistep [Figure 5G: F (2, 45) = 0.2524, $p = 0.7780$] and upward ramp calls [Figure 5H: F (2, 45) = 0.9351, $p = 0.4000$]. However, significant strain differences were observed in flat-trill [Figure 5F: F (2, 45) = 6.162, $p = 0.0043$] and complex calls [Figure 5I: F (2, 45) = 5.164, $p = 0.0096$].

Discussion

This is the first time ultrasonic calls emitted by FSL during tickling have been investigated. Our results showed that I) tickling is a suitable procedure to induce 50 kHz vocalisation in rats and II) the ultrasonic response of tickled FSL rats significantly differs from FRL and SD rats.

This study demonstrated a significant difference in the number of calls produced by the tickled rats compared to the light-touch group, which is in line with previous reports (Panksepp, 1999; Panksepp & Burgdorf, 2000; Burgdorf *et al.*, 2001; Hori *et al.*, 2013). Furthermore, when evaluating the subtypes of calls emitted, the tickled groups produced trill and flat-trill combinations, whereas the light-touch groups did not produce any flat-trill combinations and only very few trill calls. These call types were previously related to tickling (Schwarting *et al.*, 2007) and other rewarding stimuli like psychostimulants (Ahrens *et al.*, 2009; Simola *et al.*, 2010, Wright *et al.*, 2010). Furthermore, previous reports have shown that pair-tested rats presented a higher proportion of trill calls compared to single-tested rats after saline or amphetamine treatment (Wright *et al.*, 2010). Despite the rats in our study being single-housed, we observed increased calls after tickling.

Furthermore, tickled FSL rats produced significantly more calls than FRL and SD controls. This contrasts with the pattern observed in other animal models of depression, which showed fewer USV calls (Mallo *et al.*, 2009, Rao & Sadananda, 2015, Burke *et al.*, 2021).

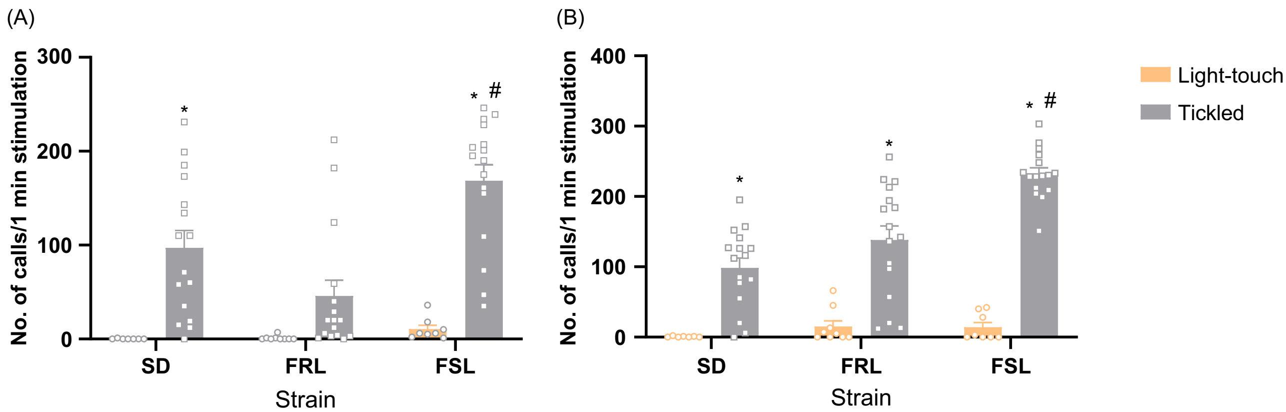

Figure 1. Differences in the no. of calls emitted by animals submitted to light-touch or tickle. (A) Number of calls on day 1 by strain. (B) Number of calls on day 23 by strain. Data presented as mean ± SEM. *$p < 0.05$ compared to light-touched of the same strain. #$p < 0.05$ compared to SD and FRL.

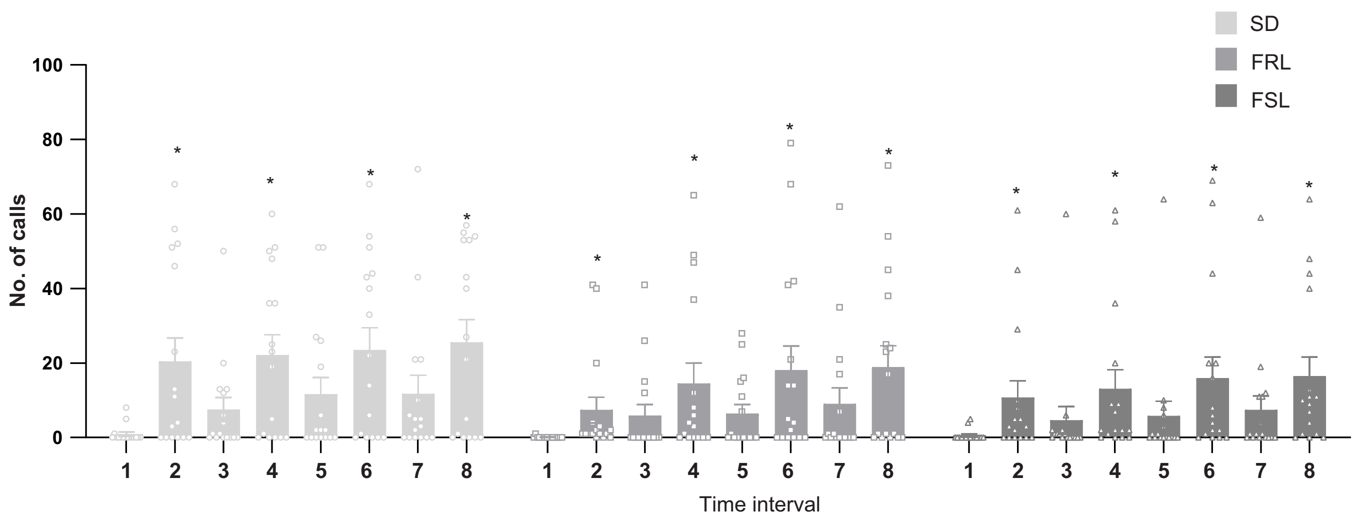

Figure 2. Distribution of 50 kHz calls between strains during tickling sessions on days 1 by interval of 15s. No stimulation intervals: 1, 3, 5, 7. Stimulation intervals: 2, 4, 6, 8. Data presented as mean ± SEM. *$p < 0.05$ compared to the previous interval.

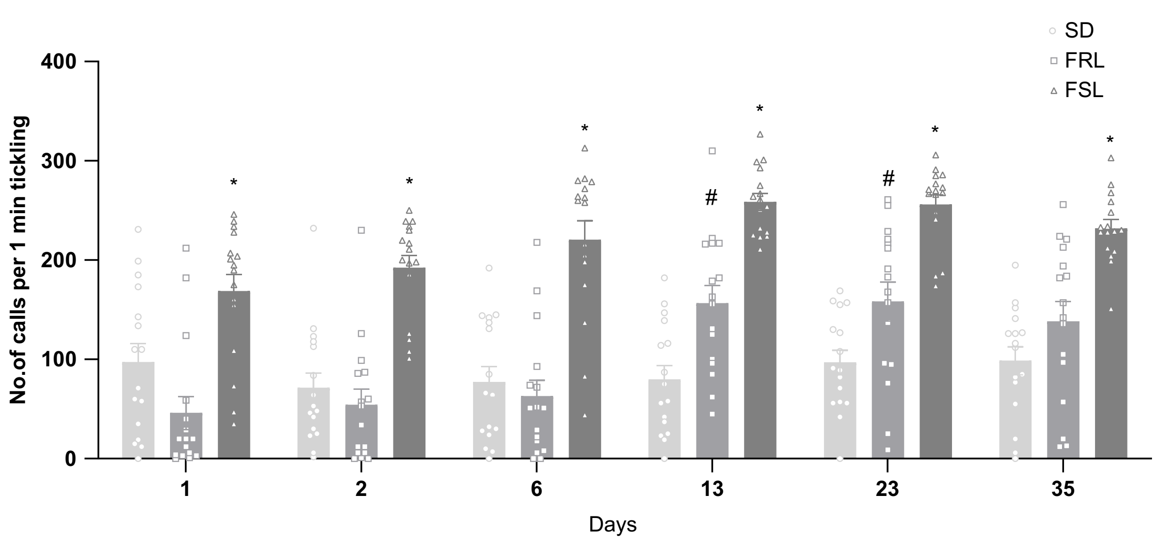

Figure 3. Distribution of calls across experimental days divided by strain. Data presented as mean ± SEM. *$p < 0.05$ FSL compared to FRL and SD, #$p < 0.05$ FRL compared to SD.

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Table 2. Stability in call profiles across days in the three strains. Correlation of calls between days across strains. FSL = Flinders Sensitive Line rat, FRL = Flinders Resistant Line rat, SD = Sprague Dawley

		Day2	Day6	Day13	Day23	Day35
FSL						
Day1	Spearmans correlation	0,658**	−0,094	0,165	0,368	0,029
	Sig. (2-tailed)	0,006	0,729	0,542	0,161	0,914
Day2	Spearmans correlation		−0,113	−0,155	0,135	0,109
	Sig. (2-tailed)		0,676	0,568	0,617	0,688
Day6	Spearmans correlation			−0,065	0,115	−0,218
	Sig. (2-tailed)			0,812	0,672	0,418
Day13	Spearmans correlation				,632**	0,483
	Sig. (2-tailed)				0,009	0,058
Day23	Spearmans correlation					0,252
	Sig. (2-tailed)					0,347
FRL						
Day1	Spearmans correlation	0,871**	0,46	−0,006	−0,102	0,037
	Sig. (2-tailed)	<0,001	0,073	0,983	0,707	0,892
Day2	Spearmans correlation		0,607*	0,258	−0,035	0,212
	Sig. (2-tailed)		0,013	0,334	0,897	0,43
Day6	Spearmans correlation			0,617*	0,331	0,244
	Sig. (2-tailed)			0,011	0,21	0,361
Day13	Spearmans correlation				0,714**	0,675**
	Sig. (2-tailed)				0,002	0,004
Day23	Spearmans correlation					0,706**
	Sig. (2-tailed)					0,002
SD						
Day1	Spearmans correlation	0,511*	0,383	0,478	0,23	0,233
	Sig. (2-tailed)	0,043	0,144	0,061	0,39	0,386
Day2	Spearmans correlation		0,524*	0,632**	0,439	0,556*
	Sig. (2-tailed)		0,037	0,009	0,089	0,025
Day6	Spearmans correlation			0,856**	0,812**	0,864**
	Sig. (2-tailed)			<0,001	<0,001	<0,001
Day13	Spearmans correlation				0,720**	0,696**
	Sig. (2-tailed)				0,002	0,003
Day23	Spearmans correlation					0,848**
	Sig. (2-tailed)					<0,001

The increase in 50 kHz calls observed in juvenile FSL suggests higher sensitivity to rewarding stimulus, which contradicts the core anhedonic state found in depression. However, animal models of depression often resemble some but not all depression features (Gururajan *et al.*, 2019). For instance, whether FSL can emulate anhedonia is a matter of controversy. Previous reports showed mixed findings in adult or prepubertal FSL (Pucilowski *et al.*, 1993; Matthews *et al.*, 1996; Malkesman *et al.*, 2005; Sanchez *et al.*, 2018). However, when exposed to specific environmental stressors, anhedonia was observed in FSL, perhaps revealing a heightened sensitivity to external stimuli (Pucilowski *et al.*, 1993). Taken together, FSL seems more sensitive to reward and aversive interventions. In agreement with the enhanced sensitivity of FSL, we found an increase in flat-trill combinations: previous data showed that flat-trill combinations induced by reward stimulus (playing, amphetamines) were only increased in pair-tested animals and not in single animals. At the same time, FSL enhanced this call type compared to the other strains (Wright *et al.*, 2010). Curiously, in humans, high environmental sensitivity during childhood may constitute a risk factor for developing depression as an adult (Lionetti *et al.*, 2022). Furthermore, the vulnerability aspects of sensitivity to reward are associated with high expression of traits such as FEAR, SADNESS and ANGER (Pulver *et al.*, 2020) of the Affective Neuroscience Personality Model (Davis &

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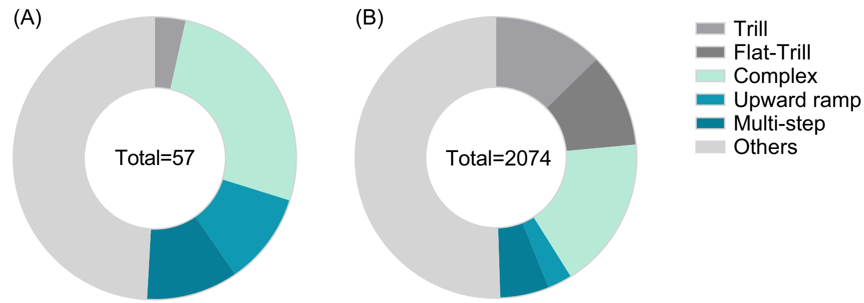

Figure 4. Qualitative analysis of calls in animals tickled or light-touch. (A) Distribution of calls in animals submitted to light-touch. (B) Distribution of calls in tickled animals. The three strains were merged in the two groups (tickled and light-touch).

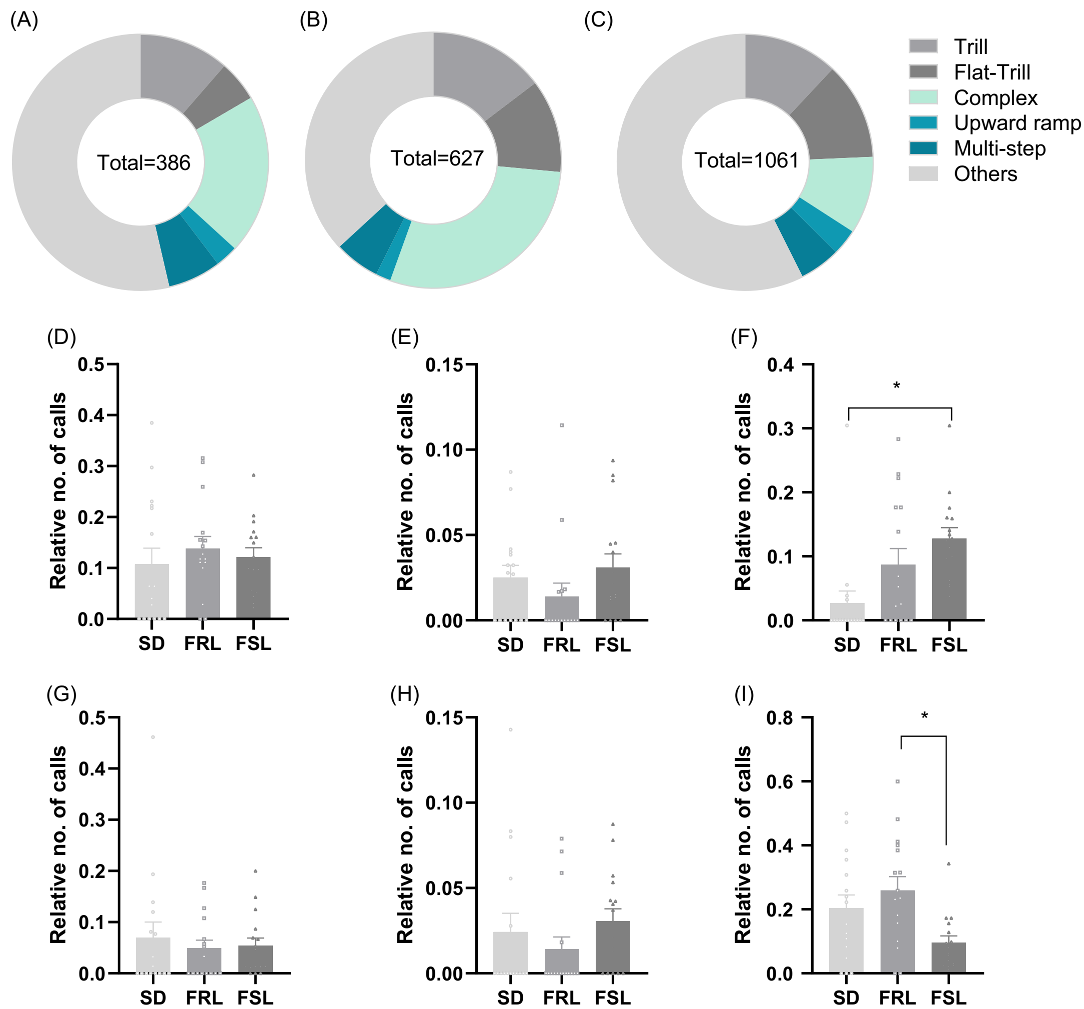

Figure 5. Qualitative analysis of calls in tickled animals by strain. (A) Calls distribution across categories in SD. (B) Calls distribution across categories in FRL. (C) Calls distribution across categories in FSL. (D) Relative quantity of trill calls in SD, FRL and FSL. (E) Relative quantity of flat calls in SD, FRL and FSL. (F) Relative quantity of trill-flat calls in SD, FRL and FSL. (G) Relative quantity of multistep calls in SD, FRL and FSL. (H) Relative quantity of upward ramp calls in SD, FRL and FSL. (I) Relative quantity of complex calls in SD, FRL and FSL. D-I Data presented as mean ± SEM. **p* < 0.05 as indicated.

Panksepp, 2011). These emotional traits serve as depression vulnerability mediators in adverse environments. Consistently, it has been demonstrated in rats that higher reward sensitivity corresponds to higher sensitivity to chronic mild stress (Koiv *et al.*, 2019). Single housing may additionally have been the stressor to precipitate 50 kHz USV response from the more reward-sensitive FSL.

Lastly, we performed a correlational analysis to determine whether the quantity of calls produced by a rat on one day was associated with the number of calls produced on later experimental days. Significant temporal stability was found for the SD rat from day 6 and the FRL rat from day 13. However, the number of calls emitted by FSL was not associated with the number of calls emitted on later days. A previous study on the temporal stability of the USVs produced during tickling stimulation in Wistar rats showed the calls from the beginning of the second week of stimulation to be associated with calls produced on later experimental days (Mallo *et al.*, 2007). The apparent instability in the number of calls

https://doi.org/10.1017/neu.2024.61 Published online by Cambridge University Press

produced between days in the FSL rat may thus be a characteristic of this specific phenotype and may relate to their reward sensitivity as a representation of depression vulnerability. One could speculate that this reflects a heightened sensitivity to the tickling stimulation. Hence, minor variations in the stimulation between days may lead to more significant differences in the number of calls produced in the FSL rats compared to the SD and FRL rats.

Thus, instead of a depressive-like phenotype, the FSL rat may present a hypersensitive phenotype, which displays a heightened sensitivity to its environment regardless of whether the stimuli are appetitive or aversive. Thus, our findings have important implications for future studies of the FSL rat within the field of depression. If the depressive-like phenotype is to be modelled, it may be advantageous to expose the FSL rat to an environmental stressor. Moreover, emotional hyperreactivity was related to bipolar disorder in patients (M'bailara *et al*., 2009, Henry *et al*., Henry *et al*., 2012). Considering the neurochemical profile and the behavioural phenotype of FSL, some authors have already suggested its potential as a preclinical tool for bipolar depression (Mncube *et al*., 2021). The hypersensitive trait may endorse this hypothesis. Future research should address the performance of FSL in bipolar-like tests such as amphetamine-induced hyperlocomotion, social interaction or their response to mood stabilisers. Moreover, combining environmental stress and tickling would be interesting to explore the USV profile of stressed FSL compared to controls.

Conclusions

The current study aimed to investigate whether USVs may be utilised as an alternative non-stressful tool to assess depressive-like behaviour in rats. The main finding of this study – a profound difference in vocalisations between an animal model of depression and controls during tickling sessions – suggests that this may be a fruitful endeavour. However, more studies on USVs in other rat models of depression are needed as the FSL rat may not be the preferred rat model to use, as it apparently shows heightened reward sensitivity and hence might not model anhedonia, at least at an early age. Instead, USVs in the FSL rat may provide a readout in a model of general vulnerability.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/neu.2024.61.

Acknowledgements.

Author contribution. Linda Marie Kai conducted all animal experiments, collected and analysed the data and made the initial draft for the article. Lia Parada Iglesias participated in the analysis and interpretation of data and the writing of the manuscript. Gregers Wegener, Kadri Kõiv and Jaanus Harro contributed to the study's conception and design, the interpretation of the data and critically revised the intellectual content of the manuscript.

Financial support. This work was supported by Region Midtjyllands Psykiatrisk Forskningsfond. Lia Parada Iglesias is supported by Horizon Europe (MSCA, PoMAM – grant 101110693). Jaanus Harro is supported by the Estonian Research Council (PRG1213).

Competing interests. Gregers Wegener was the Editor-in-Chief of Acta Neuropsychiatrica at the time of submission but was not involved and actively withdrew during this manuscript's review and decision process.

Ethical statement. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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