FGF2 Effects on Vocalizations

Effects of Neonatal FGF2 Administration on Vocalizations in Rats Susceptible to Anxiety and Depression

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Abstract

In this study we examined whether an early life intervention can permanently alter individual differences in emotional behavior, as modeled using bred high and low responder rats. We found that neonatal FGF2 administration permanently alters the emotional brain systems of individuals prone to anxious and inhibited behavior (bred low responders) as well as those animals that do not show these vulnerabilities (bred high responders). These neural changes increased positive ultrasonic vocalizations in response to experimenter induced play. The brain and behavior altering changes that result from a single neonatal dose of FGF2 could be a powerful treatment for individuals vulnerable to depression and anxiety-like behavior.

Keywords: Ultrasonic Vocalizations, Depression, Anxiety, FGF2, Low/High Responders, PAG

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Mood disorders and substance abuse result from the interactions between genetic susceptibility and environmental factors. We can model this interaction in rats by measuring "novelty seeking" or "thrill seeking" type behavior which may represent a fundamental trait that predicts a wide range of emotional and psychosocial behaviors. Rats show variable behaviors in response to a novel environment and can be selectively bred based on their exploratory locomotion into "thrill seekers" or high responders (HR) and "non-thrill seekers" or low responder (LR) groups (Piazza et al., 1989). In this study we examined whether an early life intervention can permanently alter individual differences in emotional behavior, as modeled using bred high responders (bHR) & bred low responders (bLR) rats.

Modeling individual differences in emotional reactivity using HR and LR Rats

To model individual differences in the development of emotional disorders in the laboratory we use the HR-LR paradigm which has been shown to be predictive of a range of drug-related and emotional behaviors. For instance, HR rats have greater rates of cocaine and amphetamine self-administration and have greater activity responses following exposure to the drugs (Piazza et al., 1989). The HR-LR trait also correlates with spontaneous anxiety behaviors, stress-reactivity and other measures of emotionality. HR rats show reduced levels of anxiety behavior in a number of behavioral assays including the light-dark box and elevated T-maze and also show greater wakefulness and reduced slow wave sleep. In response to chronic mild intermittent stress, bLR developed signs of anhedonia, a symptom where experiencing pleasure is lost, sooner and to a greater degree than bHR, suggesting that bLR are more susceptible to depression (Stedenfeld et al., 2011).

HR rats also have many differences in gene expression in the brain, as well as neuroendocrine functions, in comparison to LR rats (Bouyer et al., 1998; Kabbaj et al., 2000). For example, HR rats exhibit an increased and prolonged corticosterone (CORT) secretion when exposed to a novel environment (Piazza et al., 1991). The increased CORT secretion is thought to mediate behavioral functions though three modes of action. First, CORT regulates and changes neuronal activity in the brain. Second, CORT is essential to the management of homeostatic responses caused by environmental stimuli and stress. Third, dopamine neurons, which are implicated in locomotor, emotional, and motivational behavior, have CORT receptors, also known as glucocorticoid receptors (GR), on their cell bodies and are therefore modulated by CORT (Piazza et al., 1991). The increased CORT secretion is thought to be mediated by HRs' reduced levels of hippocampal glucocorticoid receptor (GR), which diminishes the GR-mediated negative feedback on CORT release (Figure 1).

Hippocampal regulation of CORT and emotional behavior is significant because major depression is associated with atrophy of brain regions, including the hippocampus. Furthermore, increased hippocampal GR has been implicated in anxiety-like behavior, as the blockade of GR receptors increased exploration and decreased anxiety-like behavior in the light-dark box for LR rats. Gene expression differences have also been found for corticotrophin-releasing factor (CRF), with stimulates CORT secretion. In HR rats, results showed greater basal CRF mRNA levels in the paraventricular nucleus and amygdala which are also important brain areas for the regulation emotional behavior (Kabbaj et al., 2000). Others report HR-LR differences in dopamine transmission in the nucleus accumbens, norepinephrine and serotonin transmission in the hippocampus, and levels of neurogenesis in the hippocampus, which are all implicated in mediating emotional behavior (Jama et al., 2008; Lemaire et al., 1999).

Given this evidence regarding novelty seeking, drug self-administration, stress responsiveness, and spontaneous anxiety-like behavior, Stead et al. suggested that HR and LR rats not only respond differently to motivating stimuli, but in fact, demonstrate fundamental differences in emotional and environmental reactivity (2006). Furthermore, differential c-fos activation, a marker of neuronal activity, in HR versus LR rats in response to psychological stress supports the claim that HRs and LRs have significant differences in emotions (Kabbaj and Akil, 2001). Differences in neuronal activity in emotional circuits is also consistent with the view that "novelty seeking" or "thrill seeking," the homologous behavior in humans, may represent a basic trait that predicts a range of emotional behaviors as seen in HR and LR rats (Stead et al., 2006; Zuckerman et al., 1979).

Individual differences in the expression of FGF2 predict emotional behavior.

A major reported difference between bLR and bHR is in hippocampal fibroblast growth factor (FGF) system, which has been implicated in mood disorders (Turner et al., 2006). Members of the FGF family have previously been shown to be dysregulated in individuals with major depression (Evans et al., 2004). According to past studies, the FGF family is known to have 22 ligands and five receptors in humans and is distributed throughout the brain. FGF1 and FGF2 are considered the prototypical ligands and are the most widely studied (Turner et al., 2006).

FGF2 causes proliferation of neural stem cells and differentiation into neuronal or glial cells at all stages of development and is found predominantly in the hippocampus but expressed in the cortex as well. (Gonzalez et al., 1995; Palmer et al., 1999; Turner et al., 2006). In FGF2 knockout mice (FGF2-/-), animals were found to have reduced amplification of neuroepithelial cells during neurogenesis, which resulted in shunted neuronal density in the cerebral cortex and

alterations in cortical cytoarchitecture (Raballo et al., 2000). Studies in neonatal FGF-/- mice showed that population size of hippocampal granule neurons were reduced but were reversed by FGF administration (Cheng et al., 2002). FGF2 also plays an important role in promoting proliferation of hippocampal neuroprogenitor cells and is up-regulated after brain injury. In FGF2-/- mice, the dentate gyrus, a region of the hippocampus, showed decreased neurogenesis in response to brain insult than control animals, which was subsequently reversed by FGF2 administration (Yoshimura et al., 2001).

It has been hypothesized that the FGF system plays a role in depression due to its effects on hippocampal neurogenesis and neuronal plasticity. Since the hippocampus projects to the emotional regions of the brain, such as the prefrontal cortex, cingulate gyrus and the amygdala, it is thought that alterations in cell proliferation underlie some of the key stress-related and emotional changes seen in anxiety and depression (Manji and Dumam, 2001). In rats, after acute stress, FGF2 is increased in limbic brain structures as well as the hippocampus (Molteni et al., 2001). In humans, FGF activity has been found to correlate with response to stress, antidepressant and antipsychotic medication, and major depression. Evans et al. found that in patients with major depression, a decreased expression of the FGF family is found in limbic structures (2004). Furthermore, this decrease is not apparent in patients treated with medications, such as SSRIs, which are shown to increase FGF2 receptors. For these reasons, it is suggested that FGF2 has neuroprotective and neurotropic properties which might act as a homeostatic response to challenges, and that a brain with reduced FGF levels has a lesser ability to respond to brain insults and therefore a greater vulnerability to anxiety and depression (Turner et al., 2009). Due to these important projections onto emotional brain regions from the

hippocampus and because FGF receptors are present in these output regions, it is hypothesized that this family of factors plays an important role in modulating depression (Turner et al., 2006).

Studies exploring the HR/LR phenotypes show that bLR rats have significantly lower levels of hippocampal FGF2 mRNA relative to bHR (Perez et al., 2009). The importance of FGF2 on drug-related behavior has been demonstrated in outbred rats which were administered FGF2 on PND2 and found to have enhanced propensity to self-administer cocaine (Turner et al., 2009). Interestingly, previous studies have shown that early life FGF2 injection decreases anxiety-like behavior (Turner et al., 2011). This evidence is particularly powerful because it demonstrates that an early life FGF2 intervention can permanently alter individual differences in emotional behavior.

Another potential area of impact for FGF2 is the periaqueductal gray, which has been implicated in influencing the emotional brain systems that mediate separation distress in animals and depression in humans (Panksepp and Watt, 2011). The PAG is regulated by oxytocin and opioids. Moreover, increased activity in this region, and potentially increases in FGF2, may potentiate negative affect (Panksepp and Watt, 2011).

Measuring the effects of neonatal FGF2 treatment on emotional behaviors in bLR and bHR using ultrasonic vocalizations

While many behavioral assays such as light-dark box and elevated T-maze provide information on anxiety-like behavior, a novel technique may provide better insight about the emotional states of rats following FGF2 treatment. Rats make ‘trills,’ high frequency vocalizations (USVs) that have been associated with the different affective states that occur during anticipation of reward or punishment (Knutson et al., 2008). Knutson et al. found that low frequency (22 kHz) calls typically indicate an anticipation of punishment or describe

avoidance behavior, whereas high frequency (50 kHz) calls indicate anticipation of a reward (2008). Furthermore, 50 kHz calls can be classified into flat and frequency modulated (FM) categories (Figure 2). More complex, FM calls have been associated with appetitive stimuli whereas simpler flat calls are observed more in social conditions that are not necessarily appetitive (Burgdorf et al., 2009). In rats, USVs have been proposed as an index of affective state, for example, during sexual encounters or exposure to drugs of abuse a greater number of FM calls are observed (Ahrens et al., 2008; Burgdorf, 2001).

Researchers have previously studied USVs as a result of heterospecific hand play ("tickling"), which is thought to activate the dopamine reward system, inducing positive affective states (Brugdorf, 2001). Heterospecific hand play mimics the rough-and-tumble play rats engage in when in social settings. This manipulation elicits 50 kHz USVs from rats, a measure of positive affect. Researchers have found individual differences in USV responses from rats, some chirping more often than others. These differences correlated with the affective behavior of rats. Mallo reports that male rats that emitted less 50 kHz calls during tickling showed greater susceptibility to stressful stimuli and showed increased exploratory behavior (2008). Therefore, USVs elicited by tickling may be a method for measuring individual differences and changes in emotional systems in response to FGF2 treatment in bLR and bHR rats.

Our study explores the long term effects of FGF2 on the neural systems of neonatal rats by measuring the USVs that young rats produce when experiencing positive affective states during tickling. We utilized heterospecific hand play with rats, which elicited positive affective states and 50 kHz calls, to study the differences in USVs between bLR and bHR rats who had

received either a single injection of FGF2 or vehicle the day after birth (PND2). After four days of hand play the rats were sacrificed and brains collected.

We predicted that there would be a strong, significant increase in the amount of flat and FM USVs produced by juvenile bLR rats while undergoing hand play following neonatal FGF2 administration. We expected that bLR rats would benefit the most from FGF2 treatment because of their lower levels of FGF2 expression and increased vulnerability to depression-like symptoms. We expected a similar trend for bHR rats; however, since bHRs already expressed relatively high amounts of FGF2 and are relatively less susceptible to depression-like symptoms, the effects of FGF2 are expected to be less pronounced than in bLRs. The overarching hypothesis of this study was that neonatal FGF2 administration would permanently alter the emotional response of individuals (bLRs) prone to anxiety and depression. These alterations would result in increased positive responses to tickling (USVs).

Methods

Subjects

High anxiety-like behavior (bLR) and low anxiety-like behavior (bHR) Sprague–Dawley rats were generated from our in-house breeding colony, where the bLR/bHR lines have been maintained for several generations (generation 32 and 33). The rats were pair housed in standard transparent cages under a controlled light cycle (12 h light/12 h dark cycle) with lights on at 06:00 h. The rats were in controlled temperature and humidity settings with food and water available *ad libitum*. The experiments followed the "Principles of Laboratory Animal Care" (http://www.nap.edu/readingroom/books/labrats/) and the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003), and are approved by the University Committee on the Use and Care of Animals (Flagel et al., 2008).

Procedure

On the day after birth (PND2), litters were culled into groups of six males and six females and injected with either FGF2 (20 ng/g in 50 μL 0.1 M PBS with 1% BSA s.c. into the axillary space; Sigma) or vehicle (0.1 M PBS with 1% BSA s.c.). FGF2 is at near maximal levels 24 h later (Turner et al., 2011).

The FGF treated bLR (13 rats) and bHR (10 rats) and control groups of bLR (11 rats) and bHR (10 rats) were given daily sessions of experimenter-induced tickling for 4 days preceded by one day of habituation on PND22. During the habituation and tickling session an animal was taken to a nearby room with dim lighting conditions (~40 lux), removed from its home cage and placed in an empty cage located under an ultrasonic detector. The habituation day included brief animal handling and a two minute period for animals to acclimate to the novel cage environment without experimenter stimulation. On tickling days, the tickling was done in accordance with Burgdorf et al. (2001), with rapid playful stimulation including pinning of the animal and finger and hand movement similar to human tickling. While the stimulation is assertive, most of the animals should not feel fear or threatened due to the touching. The ultrasound detector (NCMX-HD Neutrik) was set to a range of 0-250kHz so as to amplify the ultrasonic sounds made by the rat (Ultrasoundgate amplifier: 416H). The tickling session each day consisted of 2 minutes of stimulation, which was separated into four 15 second tickle sessions. Periods when the experimenter stimulated the animals were called: "TickleON" and periods when the animals were not being stimulated were called: "TickleOFF." The rats were then placed back into their home cages. During the hand play procedure, video was recorded and analyzed via IC Capture.

After four days of hand play the rats were sacrificed on PND27 (24 hours after the last tickle session) and brains removed. A separate set of brains were used to process FGF2 mRNA

expression in adulthood to determine whether there were long-term effects on FGF2 expression, as previously described in Turner et al. (2008).

Data acquisition

The 50 kHz range chirps were automatically measured and discriminated from foreign noises and 22kHz calls (Avisoft). 22kHz calls were visually counted by the experimenter, as the analysis software was set to filter out calls less than 25kHz due to the heavier amounts of noise in the lower frequency range. Hand scoring was visualized using a spectrogram such that sounds 10^{-77}db and above were visible. Hand scored 22kHz USV calls were defined by visually matching calls to the 22kHz calls shown in Figure 2. Calls classified as 22kHz were a minimum duration of 100ms and flat in the spectral range of 22kHz to 36kHz. This range includes calls up to 36kHz because some 22kHz flat calls were preceded by a short "ramp" component that had a higher frequency.

During the automated scoring, the Avisoft software was set to distinguish the ideal sound element separation for our calls and we found that the "automatic whistle tracking" setting best selected the animals' calls. It uses an algorithm that searches for relatively stable peak frequency courses without dramatic frequency modulations. For a sound to be counted as a USV, its maximum change in peak frequency per millisecond was set to ± 10kHz, the minimum call duration to 8msec and minimum time passed in between each call to 25msec. These settings led to a less than 5% false detection rate when compared to hand-scored files for the total USV counts. In order to further distinguish 50kHz calls from 22kHz and noise, we constructed a database that included a profile of all the calls made in the experiment and created a minimum peak frequency threshold of 36kHz. This threshold was chosen because comparisons with hand scoring indicated that this threshold reliably eliminated 22kHz calls and some residual noise.

Inspection of a peak frequency histogram for the entire database similarly indicated that 36 kHz divided low frequency and high frequency clusters (Figure 3).

To differentiate 50kHz FM calls from flat calls, we demonstrated that there was a bimodal distribution for the mean standard deviation of the peak frequency (Figure 4). We used this bimodal distribution to create a threshold cut-off at the 0.10 standard deviation (SD) mark (SD>0.10 = frequency modulated (FM), SD<0.10= flat). A comparison with hand scoring indicated that this threshold predicted FM calls with 95% accuracy. Flat calls were predicted with 75% accuracy because some multi-component FM calls only had one component detected and were artificially labeled as flat. Calls were considered flat if they matched characteristics of "flat" and "short" calls as seen in Figure 2 and all other calls were considered FM calls, except for the 22kHz USV which were hand scored.

FGF2 mRNA in situ hybridization

A separate set of brains (PND60) were used for FGF2 gene expression (bLRVEH = 8; bLRFGF2 = 7; bHRVEH = 8; bHRFGF2 = 8). Brains were then sliced at 10µm slices on a cryostat and stored at -80°C. The in situ hybridization for this experiment was previously performed by Drs. Cortney Turner and Sarah Clinton, according to methodology described in Turner et al. (2008). In general, in situ hybridization histochemistry technology measures mRNA expression inside the cells. To do this, a radioactive riboprobe ("antisense"), complementary to the mRNA in the tissue, is placed on tissue sections and hybridizes with the transcript of interest, FGF2. The amount and location of radioactivity (^{35}S molecules conjugated to ribonucleotides) that hybridizes with the local mRNA can then be measured via autoradiography, which is a manner of visualizing radioactivity using x-ray film (Carter et al., 2010). For our in situ hybridization analysis, the sequence of rat mRNA used for generating the

probes was complementary to the following RefSeq database numbers: FGF2 (NM_019305 and 716–994). The exposure time for the film for our PAG tissue was 8 weeks. Digitized autoradiographic images were quantified using NIH ImageJ software in the ventral and dorsal regions of PAG in tissue where the background used was the forceps major corpus callosum (Bregma: -7.64mm to -4.88; Paxinos and Watson, 1986). Template figures were used to outline the region of the dorsal and ventral PAG. Signal was labeled if it surpassed 3.5X the standard deviation above the mean signal for the background elements from the corpus callosum. Optical density was signal in the labeled area subtracted from the background signal (Figure 5). Four to eight samples were measured for each brain.

Data Analysis

The number of flat and FM calls on habituation and tickling days was initially analyzed using four separate 2 x 2 (group x treatment) between subjects ANOVAs. Then a more detailed ANOVA was performed of tickling days data that included the within-subjects variables of tickling day (day 1-4), tickleON/OFF periods and call type (flat vs. FM), as well as the between subjects variables of group and treatment. Nonparametric analysis (Fisher's exact test) was used for the 22kHz calls because so few animals made these calls during tickling sessions. FGF2 integrated density in the PAG was analyzed using a 2 x 2 between subjects ANOVA.

Results

Ultrasonic Vocalizations

During the habituation period, bHR rats showed a significantly greater number of flat and FM calls in comparison to bLR rats. Additionally, rats treated with FGF2 showed a significantly greater number of flat and FM calls in comparison to controls (Figure 6; Figure 7). Thus, there was a main effect of phenotype and treatment on both the number of flat and FM calls during

habituation *(phenotype on flat calls: F(1,40)=13.042, p = .01; phenotype on FM calls: F(1,40)=12.063, p = .01, treatment on flat calls: F(1,40)=5.713, p = .02, treatment on FM calls: F(1,40)=11.299, p > .01).* Our results also show bHR/bLR differences in the effect of FGF2 on flat and FM calls. bHR rats showed a greater increase in flat and FM calls when treated with FGF2 in comparison to bLR rats during habituation. Thus, during the habituation period our results also demonstrated an interaction between the effects of phenotype and FGF2 treatment on the number of flat and FM calls (*F(1,40)=5.713, p = .02; F(1,40)=7.263, p = .01, respectively).*

During all the tickling days combined, similar results were found. bHR rats showed a greater number of flat and FM calls in comparison to bLR rats and animals treated with FGF2 showed a significantly greater number of flat calls in comparison to controls (Figure 8; Figure 9). There was a main effect of phenotype on both the number of flat and FM calls and a main effect of treatment on flat calls during tickling periods *(phenotype on flat calls: F(1,40)=18.536, p < .01; phenotype on FM calls: F(1,40)=15.612, p < .01; treatment on flat calls: F(1,40)=5.919, p = .02).* Our results also showed bHR/bLR differences on FM calls. bLR rats showed greater increases in FM calls when treated with FGF2 in comparison to bHR rats during tickling. Thus, our results demonstrate an interaction between the effects of phenotype and FGF2 treatment on the number of FM calls during tickling *(F(1,40)=5.218, p = .03).*

A more detailed analysis of USV calls across tickling days and while comparing tickleON and tickleOFF periods also showed interesting results. The total numbers of USV calls changed across tickling days especially for the bHR-FGF2 group. The bHR-FGF2 group showed an increase in calls with a peak on the final tickle day (Figure 10). Thus, there was a main effect of day on the total number of USV calls, an interaction between day and group on total number of USV calls and an interaction between day, group and treatment on total number of USV calls

(F(2.538, 40)=4.491, p = .01; F(2.538, 40)=5.427, p > .01; F(2.538, 40)=8.930, p < .01, respectively). A similar trend is observed when examining tickleON and tickleOFF sessions. In general, animals showed a greater number of calls during tickleON periods in comparison to tickleOFF, and bHR-FGF2 rats showed a greater number of flat and FM vocalizations during tickleON and tickleOFF periods as tickling days progressed, while the other groups did not show significant changes (Figure 11; Figure 12). There was a main effect of tickleON vs. tickleOFF on the total number of USV calls and an interaction between tickleON vs. tickleOFF and group *(F(1, 40)=332.9, p < .01; F(1,40)=20.550, p < .01, respectively).*

Figures 11 and 12 also show notable differences in the distribution of flat and FM calls. In general, animals showed a greater number of flat calls than FM calls, while bHR rats and FGF2 rats show an increased proportion of FM calls to flat calls in comparison to bLR and control rats. Interestingly, as tickling days continued the bHR-FGF2 rats, in particular, showed a marked increase in the proportion of FM calls to flat calls. There is a main effect of call type on total USV calls as well as an interaction between call type and group and treatment, respectively, on total USV calls *(call type on total USV calls: F(1, 40)=313.142, p < .01; interaction between call type and group on USV calls: F(1, 40)=10.046, p > .01; interaction between call type and treatment on USV calls F(1, 40)=5.172, p = .03).* There was also an interaction between day, FM and flat calls, group, and treatment on total USV calls *(F(2.740, 109.589)=91.594, p < .01).*

Figure 13 shows the proportion of animals that made 22kHz USV calls during recording. Significant differences between phenotype or treatment were not observed most likely due to the small numbers of 22kHz calls emitted overall (*p = 1.0; p = .14, respectively).*

FGF2 In Situ Hybridization

Figure 14 and 15 shows results for FGF2 mRNA expression in the dorsal and ventral periaqueductal grey (PAG), respectively. There were not any significant differences in the expression of FGF2 in any area of the PAG between the two phenotypes or two treatment groups *(phenotype on average optical density (AOD) in the ventral PAG: $F(1,27)=1.339$, $p = .26$; phenotype on AOD in the dorsal PAG: $F(1,27)=1.135$, $p = .30$; treatment on AOD in the ventral PAG: $F(1,27)=0.038$, $p = .85$; treatment on AOD in the dorsal PAG: $F(1,27)=0.013$, $p = .91$; interaction of phenotype and treatment on AOD in the ventral PAG: $F(1,27)=1.884$, $p = .18$; interaction of phenotype and treatment on AOD in the dorsal PAG: $F(1,27)=0.002$, $p = .96$).*

Discussion

Our hypothesis that neonatal FGF2 administration permanently alters the emotional response of individuals (bLRs) prone to anxiety-like and depression-like behavior was supported by the results of our study. Moreover, early life treatment with FGF2 increased positive affect in response to hand play, as measured by 50 kHz USVs. Thus, a single early life intervention can permanently alter emotional response in vulnerable animals.

While we did not find significant changes in the expression of FGF2 mRNA in the ventral or dorsal PAG, many other areas of the brain may have been permanently altered, such as the hippocampus, as was previously shown in Turner et al. (2011). Another interesting result was that FGF2-bHR rats showed a potentiation in 50kHz USV production as tickling days progressed, suggesting a possible sensitization to the rewarding environment. This is similar to results found in other papers where bHR animals showed greater sensitization to drugs of abuse over time (García-Fuster et al., 2009). Interestingly, drugs of abuse also impact brain regions that play a role in USV production, depression, and anxiety behavior (Cryan and Holmes, 2005).

This finding is profound in that it is also one of the few times that our lab has seen bHR animals respond to FGF2.

While USV data from the tickling days showed significant differences between groups, the habituation day might give insight to an interesting phenomenon. The habituation day showed significantly greater USVs among bHR in comparison to bLR, whereas tickling days did not show as pronounced of a difference in USVs between bHR and bLR. This raises the question as to whether a ceiling effect in the number of USVs an animal can make is being observed for the bHRs on tickling days. Since such a high number of USVs were already being made, it is possible that the rats cannot physically make any more USVs in 2 minute session by bHRs and that the number of USVs reached a plateau due to these motor limits.

Alternatively, our results could be interpreted as FGF2 altering the brain regions involved in locomotor behavior, and, therefore USVs were increased as a by-product of increased motor movement. Some researchers suggest that USVs are simply increased as breathing increases or when thoracic compression is induced in rats. This theory would be supported by the fact that HR rats have greater locomotion and USV calls (Blumberg, 1992; Stead et al., 2006). Perhaps the mechanisms of tickling are not fully understood, but our results unequivocally reveal a correlation between USVs with FGF2 treatment. We still feel our original interpretation is still valid, because while USVs are influenced by locomotor behavior, USVs are still very tightly correlated to changes in the neurobiology of emotional brains systems (Ahrens et al., 2009; Mu et al., 2010). Furthermore, our results show changes in the ratio of flat calls to FM calls suggesting that USVs are indeed a useful assay for measuring emotional behavior changes in rats. With these principles, we feel that we can reach our conclusions based on a foundation of research.

One weakness of this study is the nature of the tickling experience. Since it is the individual experimenter who is engaged in the tickling, every tickling experience cannot be replicated exactly, especially when taking into account the amount of touches and flips the experimenter completes. Tickling is also influenced by the behavior of the rat. If the animals show signs of distress, the experimenter is less able to make tickling contact than if the animal is excited and engaging. Consequently, a possible mechanism of FGF2 effects on USV production could be that FGF2 altered how animals solicited tickling, which then changed the amount and quality of tickling induced, causing a change in USV count and type. To fully address the mechanism of FGF 2 treatment, another area of follow-up investigation includes analysis of behavior video recorded during tickling experimentation to match exact tickleON and tickleOFF timings to USV sonograms, as well as the analysis of other behaviors, such as orienting or freezing movements. Our follow up work includes analyzing film of the tickling session videos to determine if FGF2 animals solicit tickling more than controls. Potentially, in the future, we could address these weaknesses by designing a study that does not use tickling to elicit USVs. For instance, measuring USVs during rewarding situations such as cocaine self-administration could give us similar insight. However other rewarding situations could result in lesser overall USV calls, especially in adults which would give us less robust data (Kelly and Flagel, unpublished data). Another weakness of our study includes the automated analysis of USV data. While only 75% of flat calls were accurately identified, FM calls still saw the same trend in changes betweens groups, which were identified at 95% accuracy. In the future, using hand counting or further developing our technology to be able to perform more in depth analysis of call type would give us even more accurate and robust data.

These results raise several questions. For instance, understanding how and where FGF2 acts on the brain to determine behavior in animals is important. One possible mechanism of FGF2 is effects on call production that permanently alter the brain at a young age and this causes changes in adult social behavior. Another possible theory is that FGF2 alters neonatal interactions with the mother, which, in turn, alters their social behavior and social brain regions which propagate altered adult social behavior, including anxiety-like behavior (Cortney et al., 2011). Further localizing where FGF2 acts on the brain following injection on PND1, and what other behaviors it potentially impacts during early development, such as pups' interactions with mothers, should be studied in the future. Other brain regions besides the PAG that could be examined during the juvenile period or early development include the nucleus accumbens and the amygdala. Moreover, gaining a better general understanding the significance of USVs elicited from tickling is important.

Researchers now have a novel tool to assess for neurobiological differences in bLRs and bHRs. Furthermore, the long term implications of a single early life intervention altering emotional brain systems, vulnerability to depression, and addictive behavior, are enormous. The brain and behavioral changes that result from a single neonatal dose of FGF2 could be a powerful treatment for individuals vulnerable to depression and anxiety-like behavior.

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Figure Captions

Figure 1. Stress Axis. The figure illustrates the regulatory mechanisms of key emotional areas of the brain, including the hippocampus. In the hypothalamus, the paraventricular nucleus releases CRF, which is transported to the anterior pituitary, where it causes the release of ACTH into the blood stream. ACTH stimulates the adrenal cortex to synthesize and release the CORT. Glucocorticoids feed back at the level of the hippocampus, hypothalamus and pituitary to dampen excess activation of the stress axis (adapted from Hyman, 2009).

Figure 2. Representative calls for each of the categories of 50-kHz USVs (**a**) and a 22-kHz USV (**b**). Several exemplar FM calls are shown for each 50-kHz call category and one exemplar flat call is shown; these examples are not necessarily consecutive nor made by the same rat. The time scale for all 50-kHz calls is indicated in the top left panel (adapted from Wright et al., 2010).

Figure 3. Distribution of the mean peak frequencies of USV calls. This histogram indicates the overall frequency of occurrences for the mean peak frequencies for all USV calls. The x-axis indicates the mean peak frequencies and the y-axis indicates the number of occurrences for each mean peak frequency. The red line indicates the cut-off frequency at 36kHz used to separate 50kHz calls from non calls.

Figure 4. Distribution of the standard deviations for the peak frequencies of USV calls. This histogram indicates the overall frequency of occurrences for the standard deviations of the peak frequencies for all USV calls. The x-axis indicates standard deviation of peak frequencies and the y-axis indicates the number of occurrences for each standard deviation. The red line indicates the threshold cut-off used to separate flat calls from FM calls at 0.1 standard deviations.

Figure 5. Sample autoradiographs and the templates used for the dorsal and ventral PAG. This figure presents autoradiographs illustrating FGF2 labeled coronal brain slices. Yellow lines indicate the templates used to outline (**a**) the dorsal and (**b**) ventral PAG for in-situ hybridization analysis.

Figure 6. bHRs and FGF2 treated groups showed greater 50kHz flat USV than controls on the habituation day. This graph indicates significant differences in the number of 50kHz mean flat USV calls between the phenotypes and treatment groups as well as an interaction between phenotype and treatment ($p < .05$). The x-axis indicates the treatment (FGF2 or vehicle) and the y-axis indicates the groups' mean USV count, the phenotype groups are indicated in the key and the standard error of the mean is indicated on the figure with error bars (±SEM).

Figure 7. bHRs and FGF2 treated groups showed greater 50kHz FM USV than controls on the habituation day. This graph indicates significant differences in the number of 50kHz mean FM USV between the phenotypes and treatment groups as well as an interaction between phenotype and treatment ($p < .05$). The x-axis indicates the treatment (FGF2 or vehicle) and the y-axis indicates the groups' mean USV count, the phenotype groups are indicated in the key and the standard error of the mean is indicated on the figure with error bars (±SEM).

Figure 8. bHRs and FGF2 treated groups showed greater 50kHz flat USV than controls on the tickling days. This graph indicates significant differences in the number of 50kHz mean flat USV calls between the phenotypes and treatment groups ($p < .05$). The x-axis indicates the treatment (FGF2 or vehicle) and the y-axis indicates the groups' mean USV count, the phenotype

groups are indicated in the key and the standard error of the mean is indicated on the figure with error bars (±SEM).

Figure 9. bHRs and FGF2 treated groups showed greater 50kHz FM USV than controls on the tickling days. This graph indicates significant differences in the number of 50kHz mean FM USV calls between the phenotypes as well as an interaction between phenotype and treatment ($p < .05$). The x-axis indicates the treatment (FGF2 or vehicle) and the y-axis indicates the groups' mean USV count, the phenotype groups are indicated in the key and the standard of the mean is indicated on the figure with error bars (±SEM).

Figure 10. The mean number of 50 kHz USV increases across tickling days for the bHR-FGF2 group. This graph indicates overall differences in USV calls between bHR and bLR rats ($p < .05$). T he x-axis indicates the day of tickling, the y-axis indicates the groups' mean USV count, the groups are indicated in the key and the standard error of the mean is indicated on the figure with error bars (±SEM).

Figure 11. FGF2 rats showed increased USV FM call counts across days during TickleON sessions in comparison to USV flat calls. This graph indicates overall changes in USV calls between bHR and bLR rats ($p < .05$). The x-axis indicates the day of the TickleON session, the primary y-axis indicates the mean flat USV count and the secondary y-axis indicates the mean FM USV count; the standard error of the mean is indicated on the figure with error bars (±SEM).

Figure 12. bHR-FGF2 rats showed increased USV FM call counts across days during TickleOFF sessions in comparison to USV flat calls. This graph indicates overall changes in USV calls between bHR and bLR rats ($p < .05$). The x-axis indicates the day of the TickleOFF session, the primary y-axis indicates the mean flat USV count and the secondary y-axis indicates

the mean FM USV count; the standard error of the mean is indicated on the figure with error bars (±SEM).

Figure 13. No differences in proportion of 22kHz USV calls are observed between groups ($p > .05$). The x-axis indicates the group and the y-axis indicates the proportion of animals per group that made 22kHz USV.

Figure 14. There was no difference in FGF2 mRNA expression in the dorsal PAG ($p > .05$). The x-axis indicates the treatment group and the y-axis indicates average optical density; for FGF2 the standard error of the mean is indicated on the figure with error bars (±SEM).

Figure 15. There was no difference in FGF2 mRNA expression in the ventral PAG ($p > .05$). The x-axis indicates the treatment group and the y-axis indicates average optical density for FGF2; the standard error of the mean is indicated on the figure with error bars (±SEM).

Figures

Figure 1.

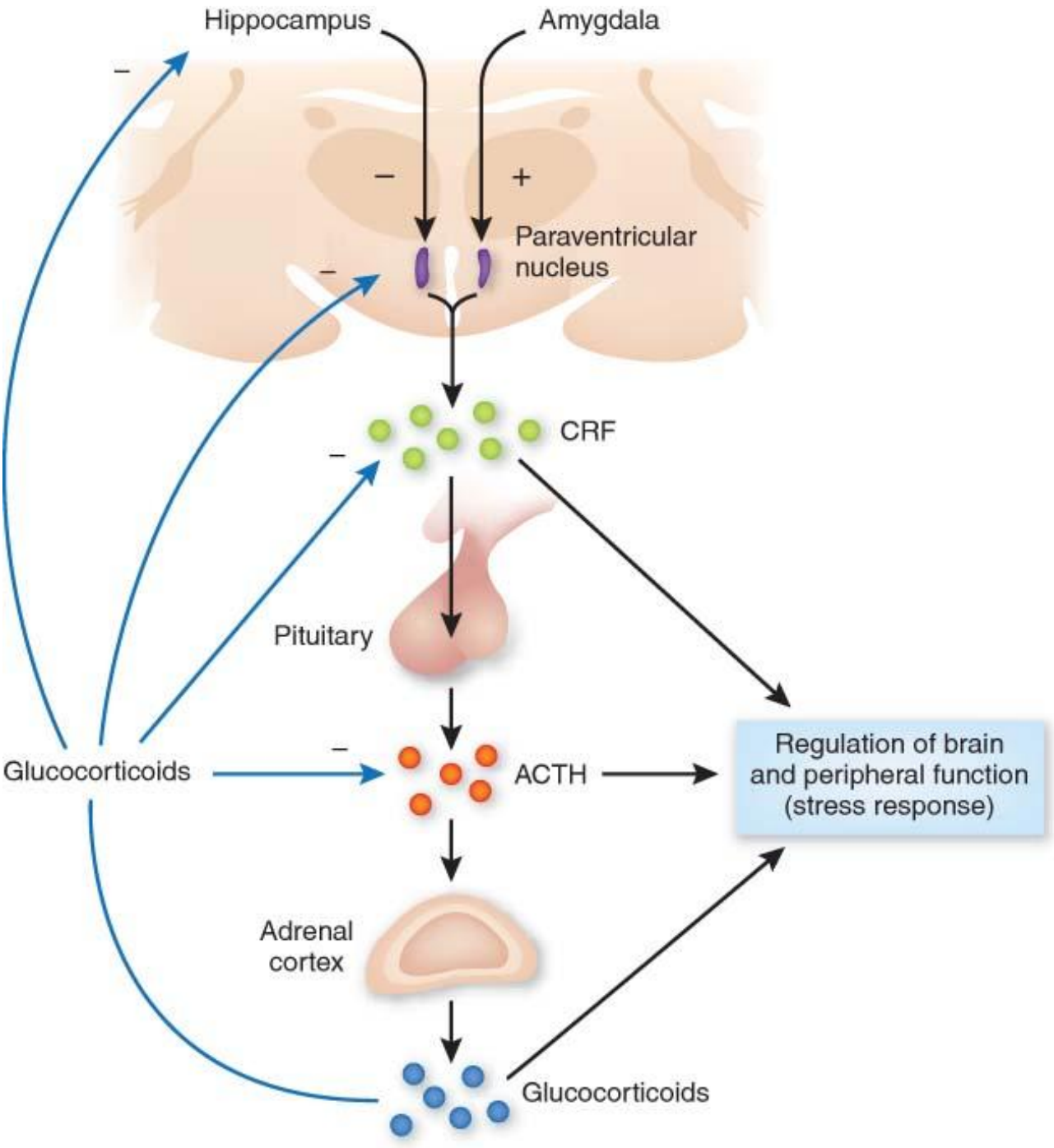


Figure 2.

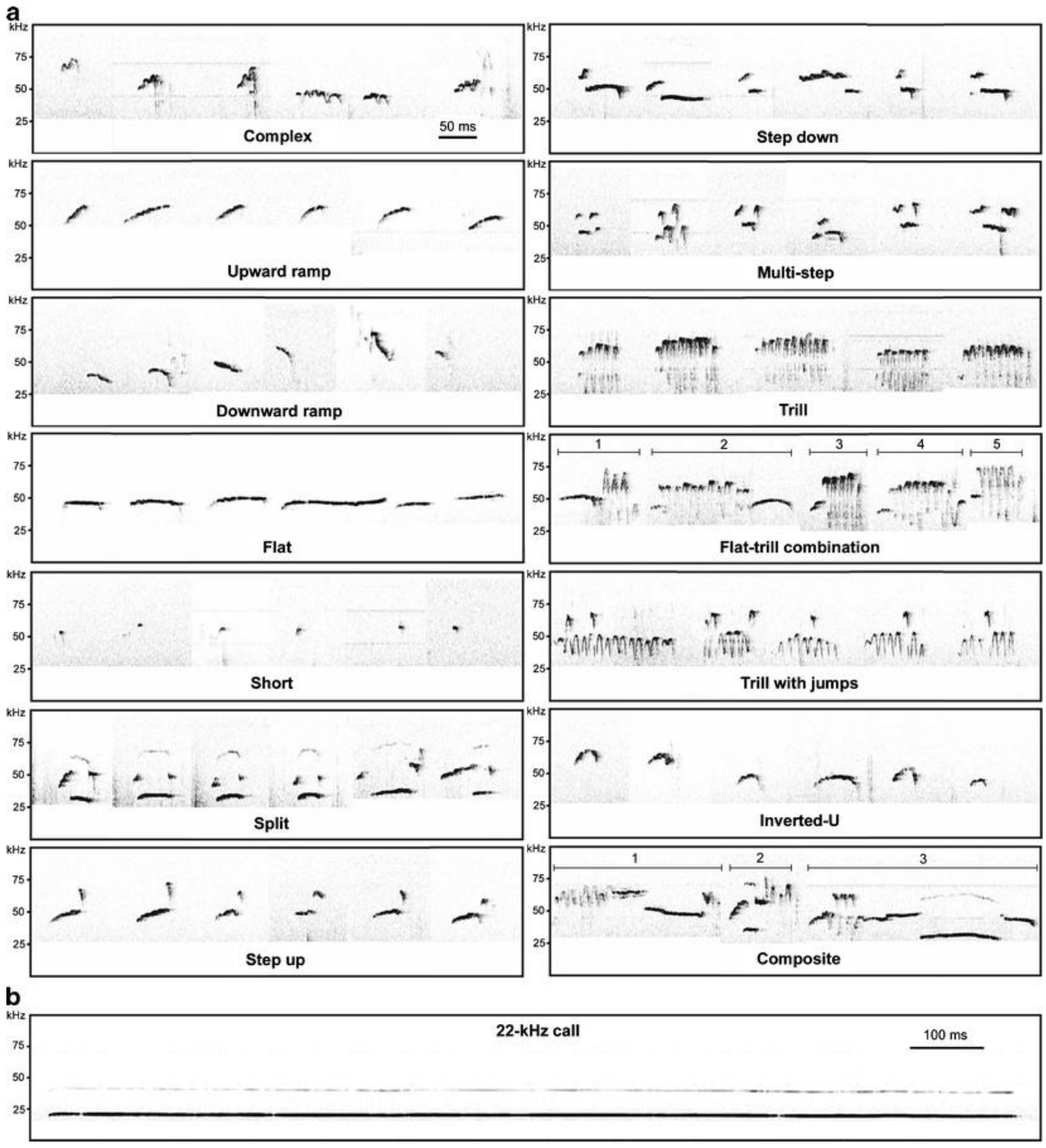
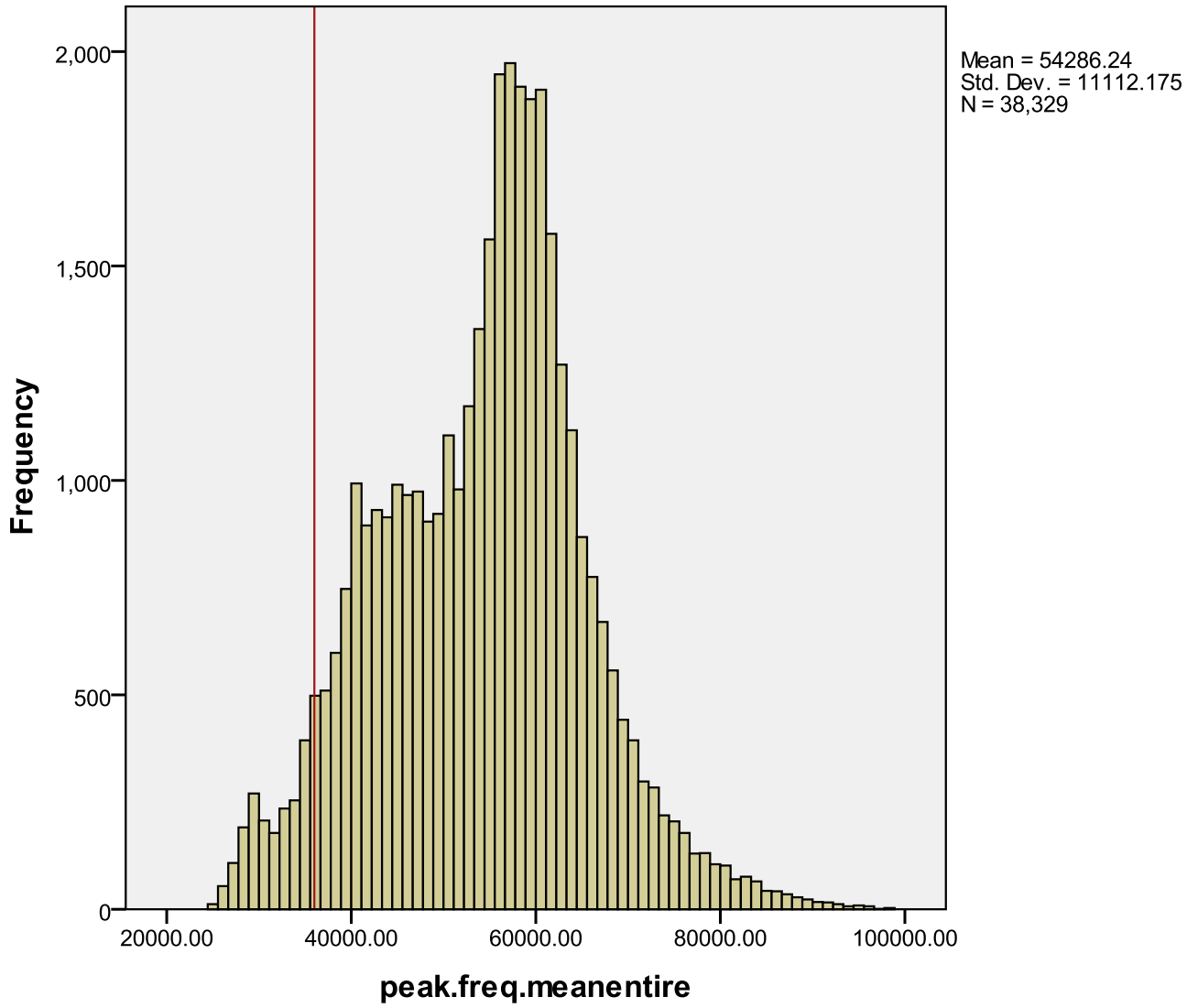

a
kHz
75
50
25
Complex
50 ms
Step down
Upward ramp
Multi-step
Downward ramp
Trill
Flat
1
2
3
4
5
Flat-trill combination
Short
Trill with jumps
Split
Inverted-U
Step up
Composite
b
22-kHz call
100 ms

Figure 3.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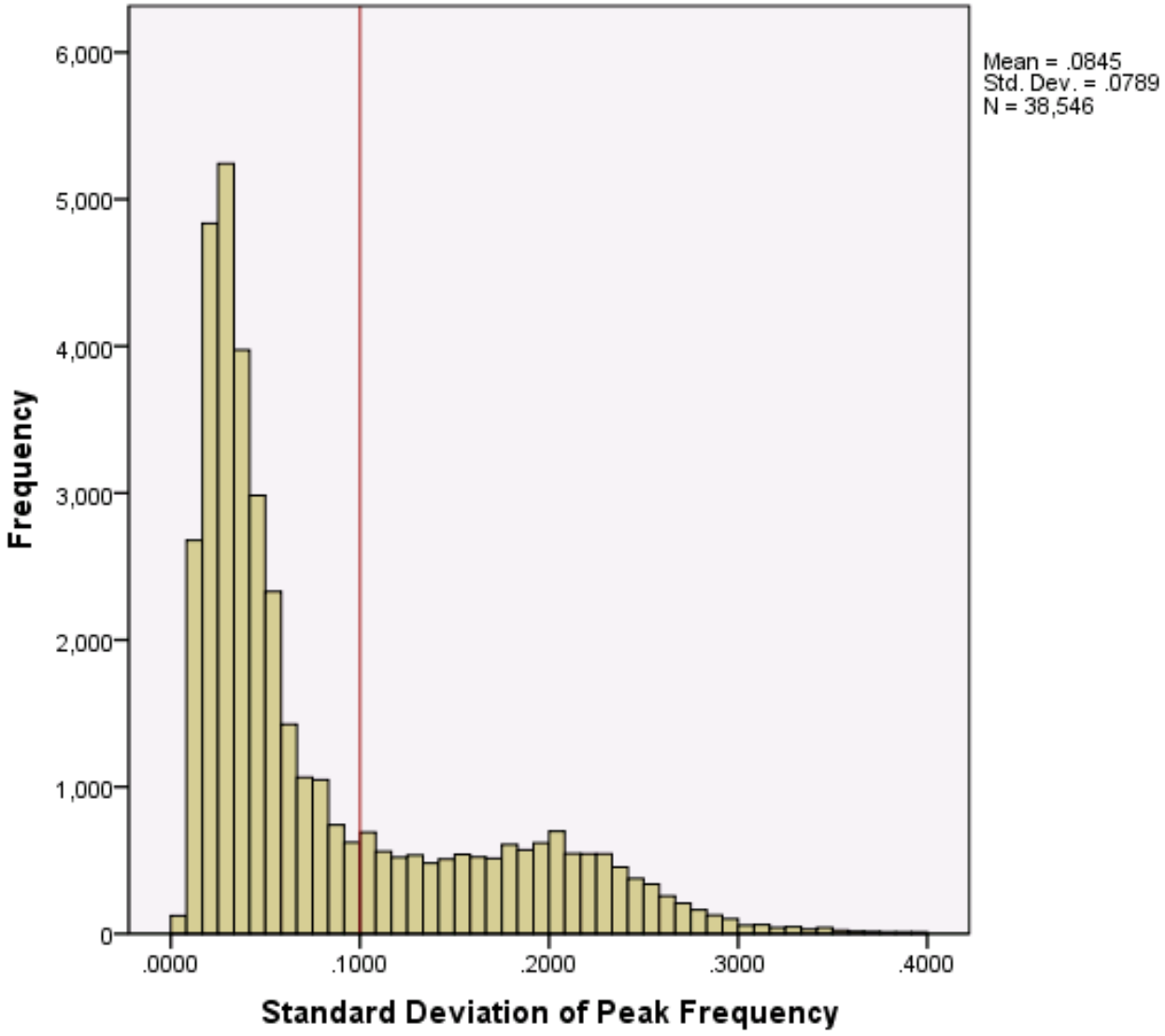
Std. Dev. = 11112.175
N = 38,329
Frequency
2,000
1,500
1,000
500
0
20000.00
40000.00
60000.00
80000.00
100000.00
peak.freq.meanentire

Figure 4.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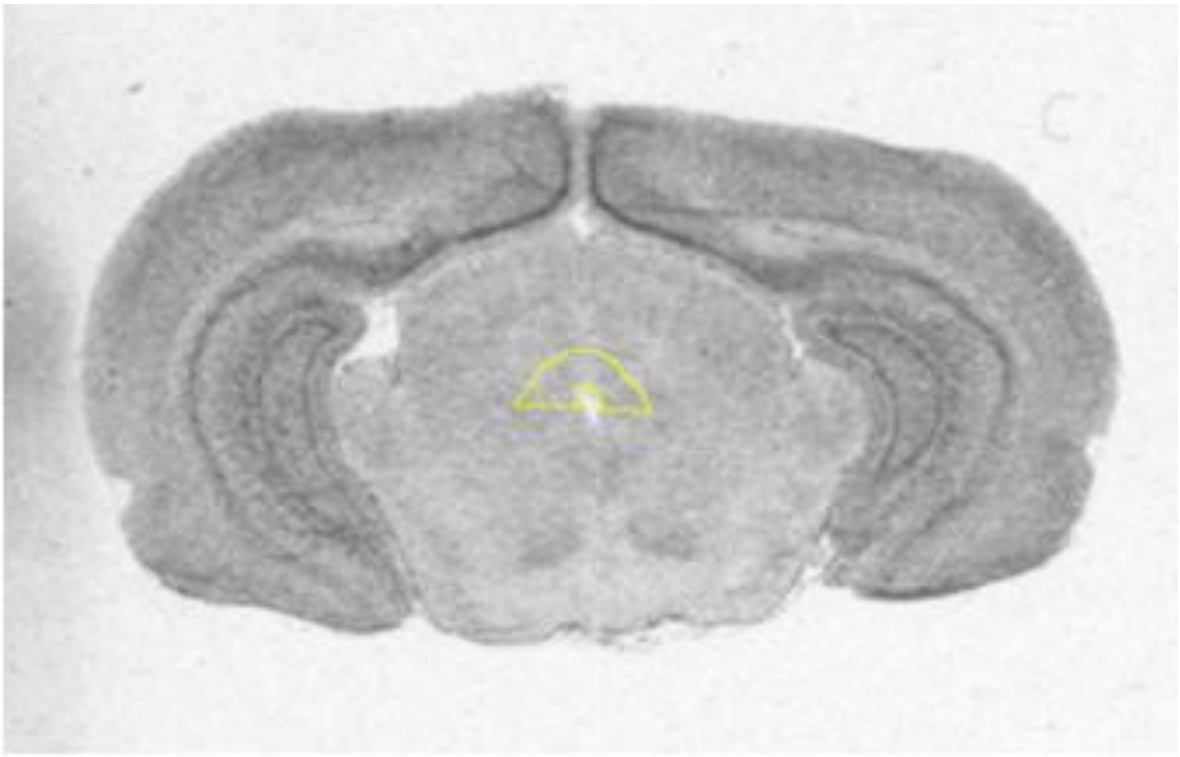
Std. Dev. = .0789
N = 38,546
Frequency
6,000
5,000
4,000
3,000
2,000
1,000
0
.0000
.1000
.2000
.3000
.4000
Standard Deviation of Peak Frequency

Figure 5.

A.



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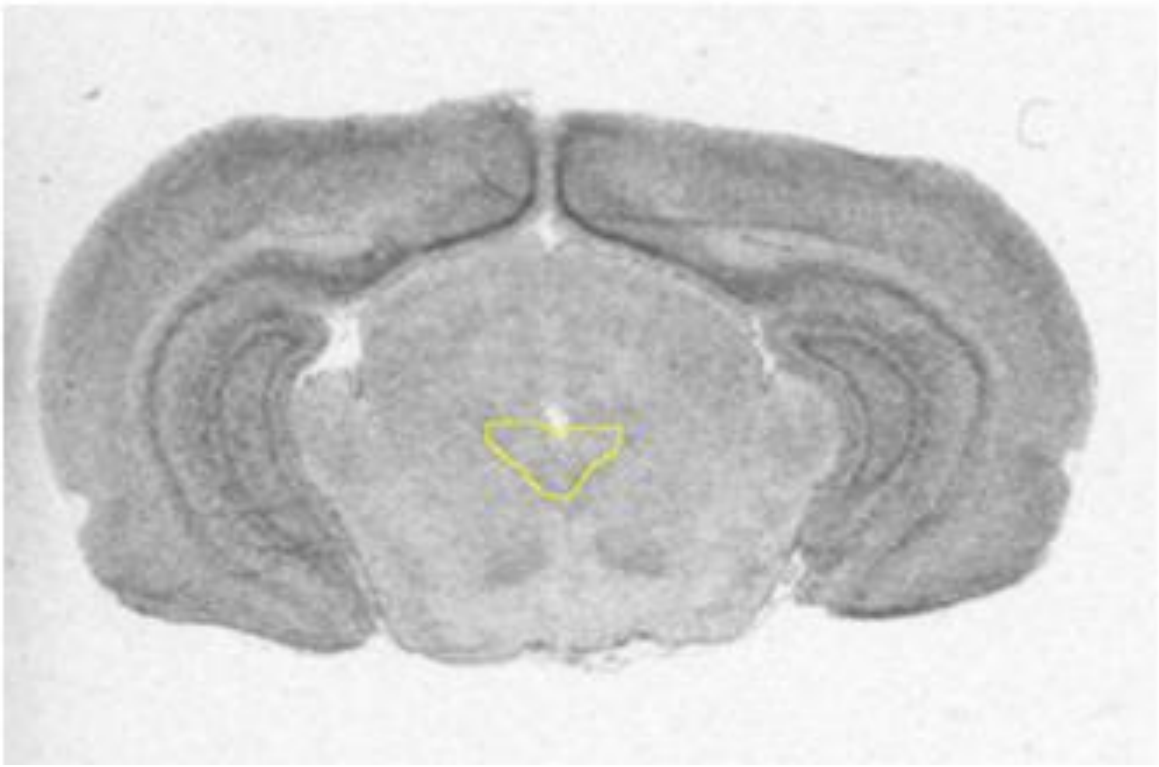


Figure 6.

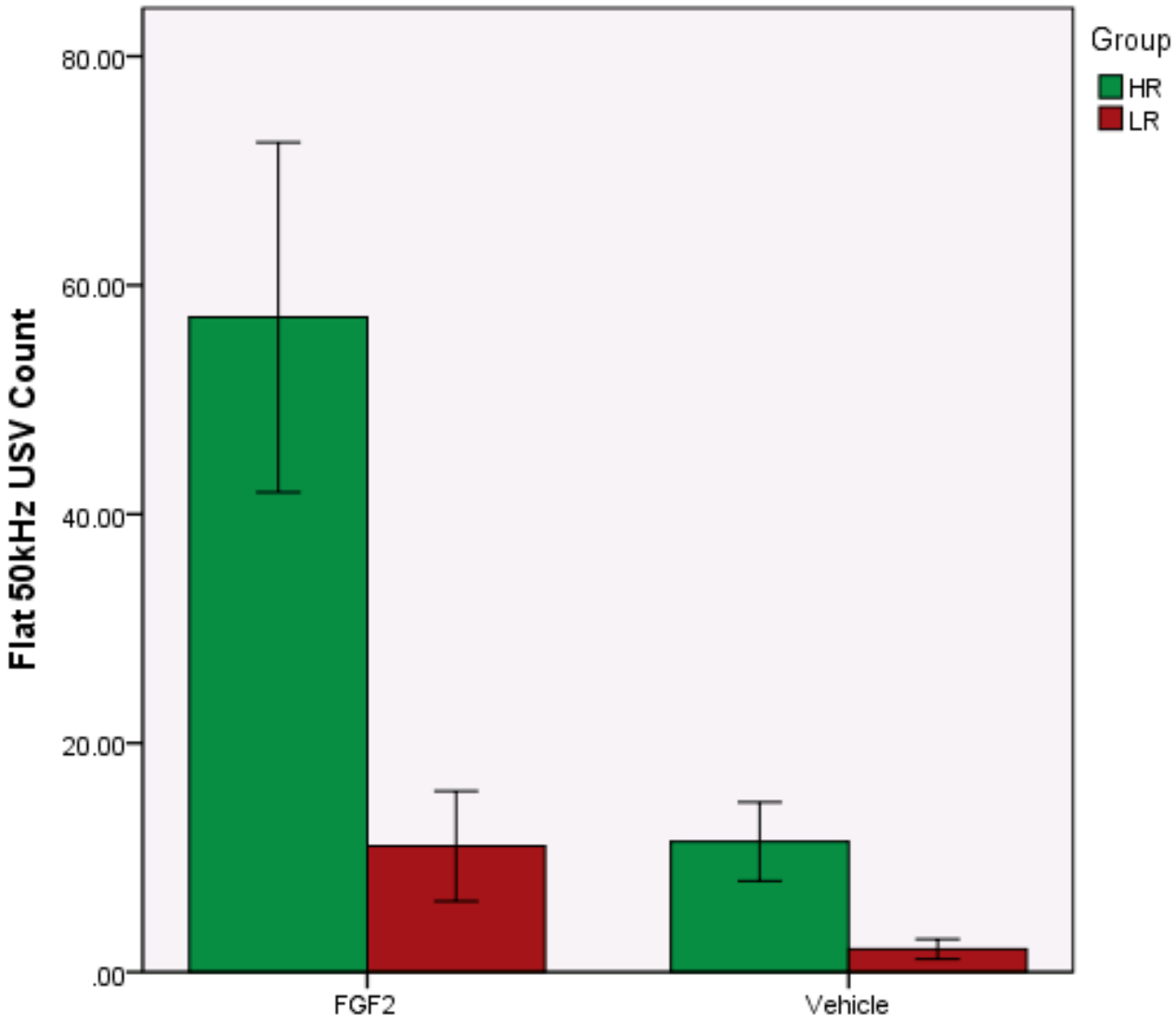

Group
HR
LR
Flat 50kHz USV Count
80.00
60.00
40.00
20.00
.00
FGF2
Vehicle

Figure 7.

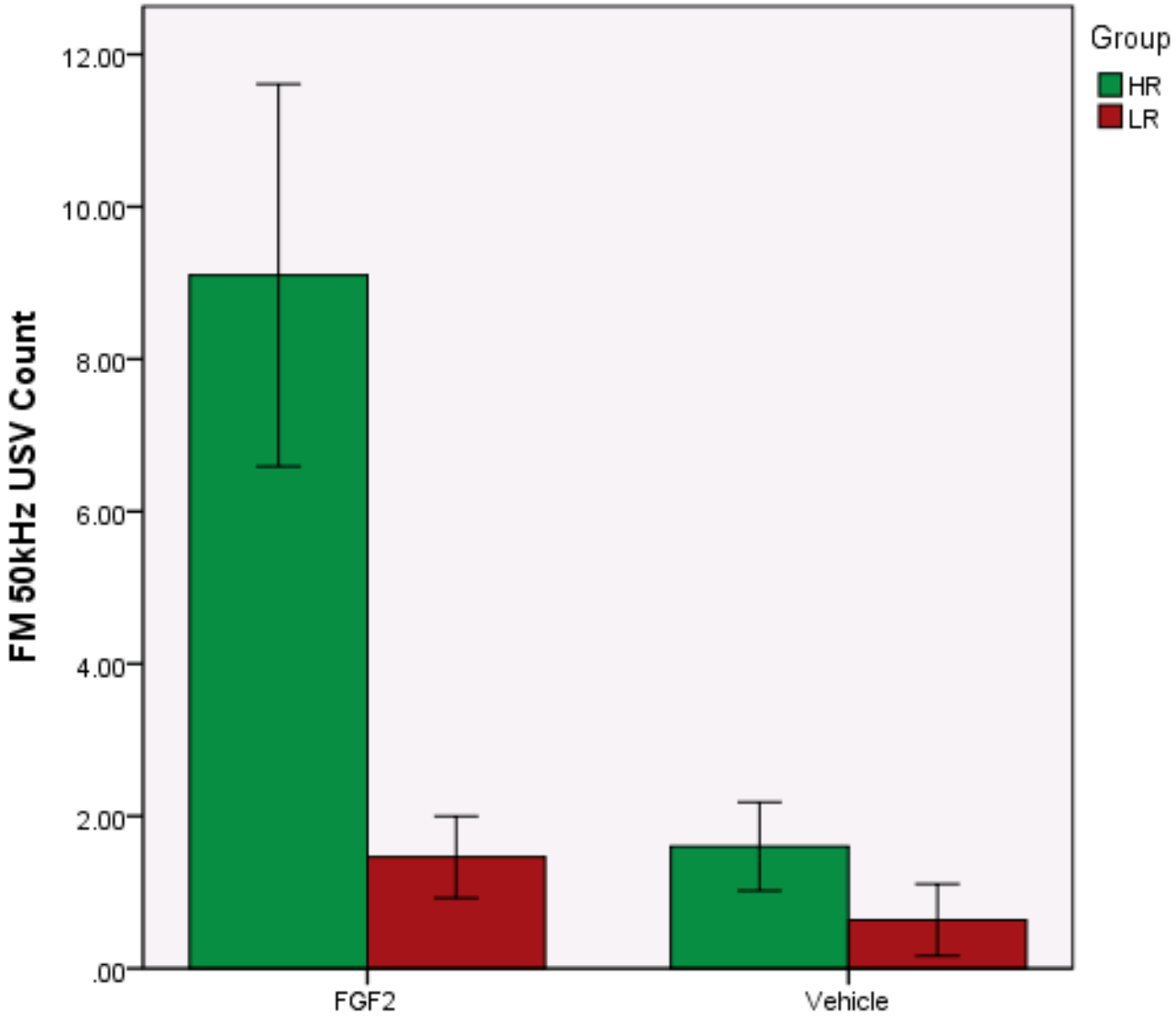

Group
HR
LR
FM 50kHz USV Count
12.00
10.00
8.00
6.00
4.00
2.00
.00
FGF2
Vehicle

Figure 8.

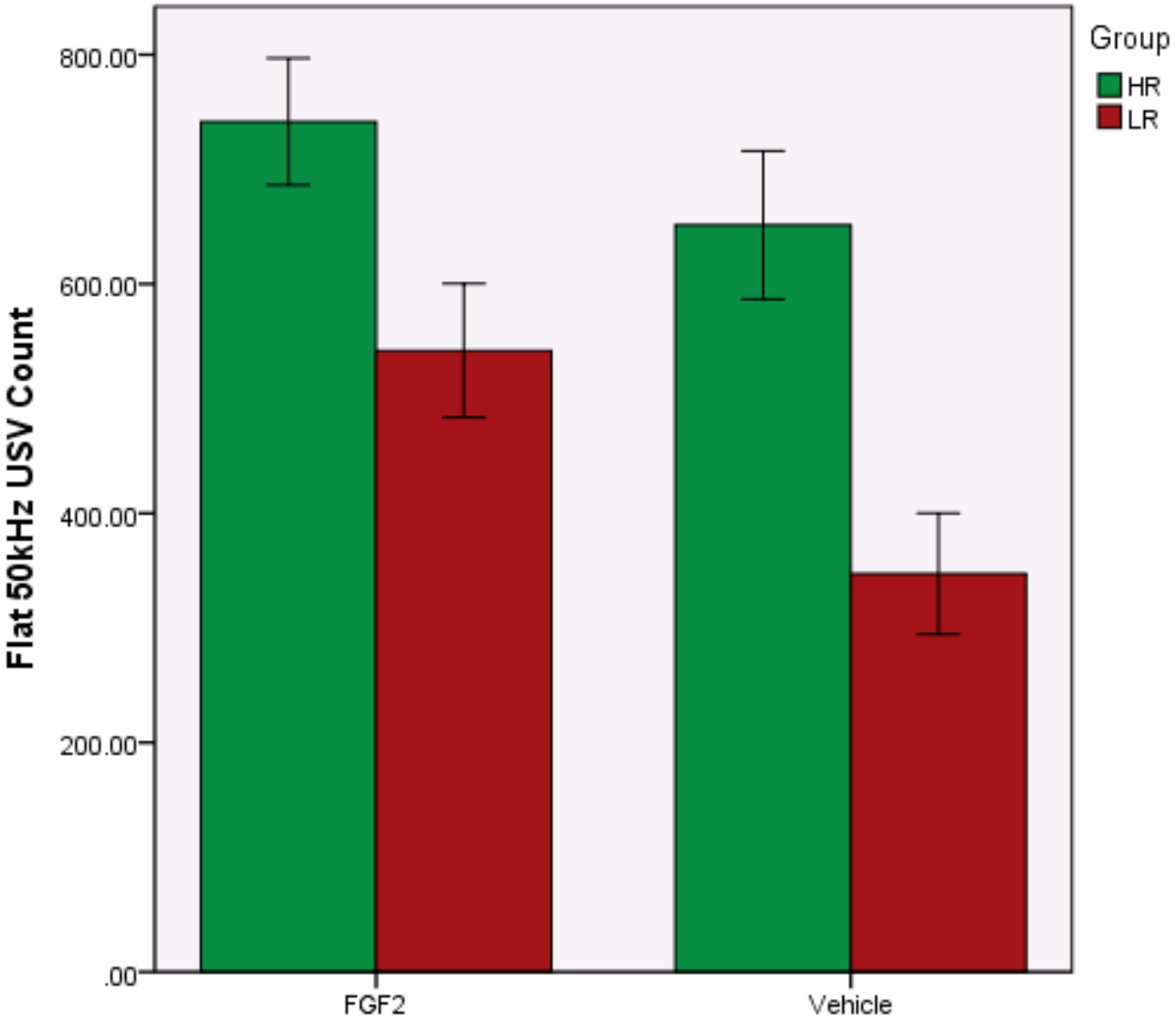

Group
HR
LR
Flat 50kHz USV Count
800.00
600.00
400.00
200.00
.00
FGF2
Vehicle

Figure 9.

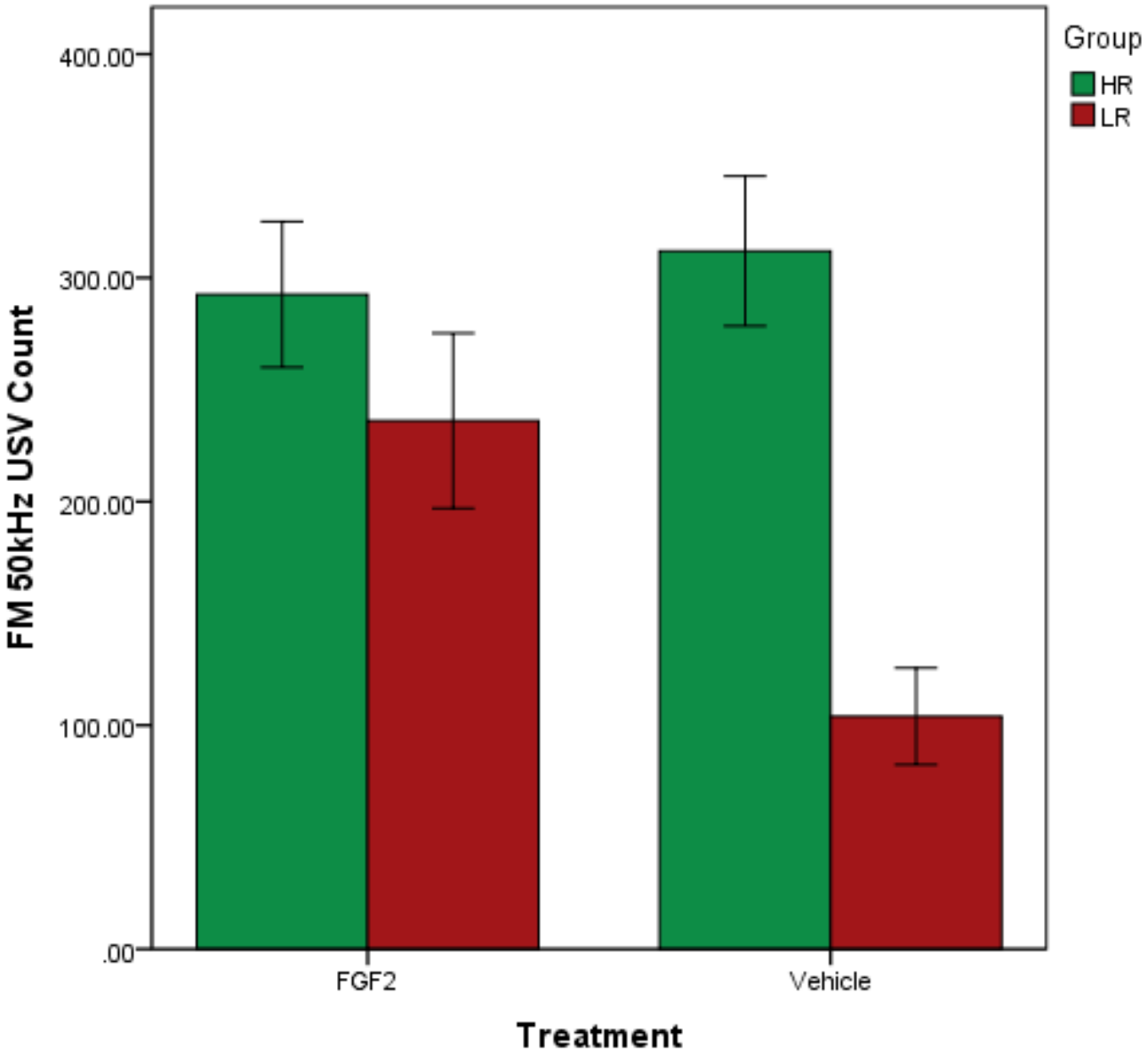

Group
HR
LR
400.00
300.00
200.00
100.00
.00
FM 50kHz USV Count
FGF2
Vehicle
Treatment

Figure 10.

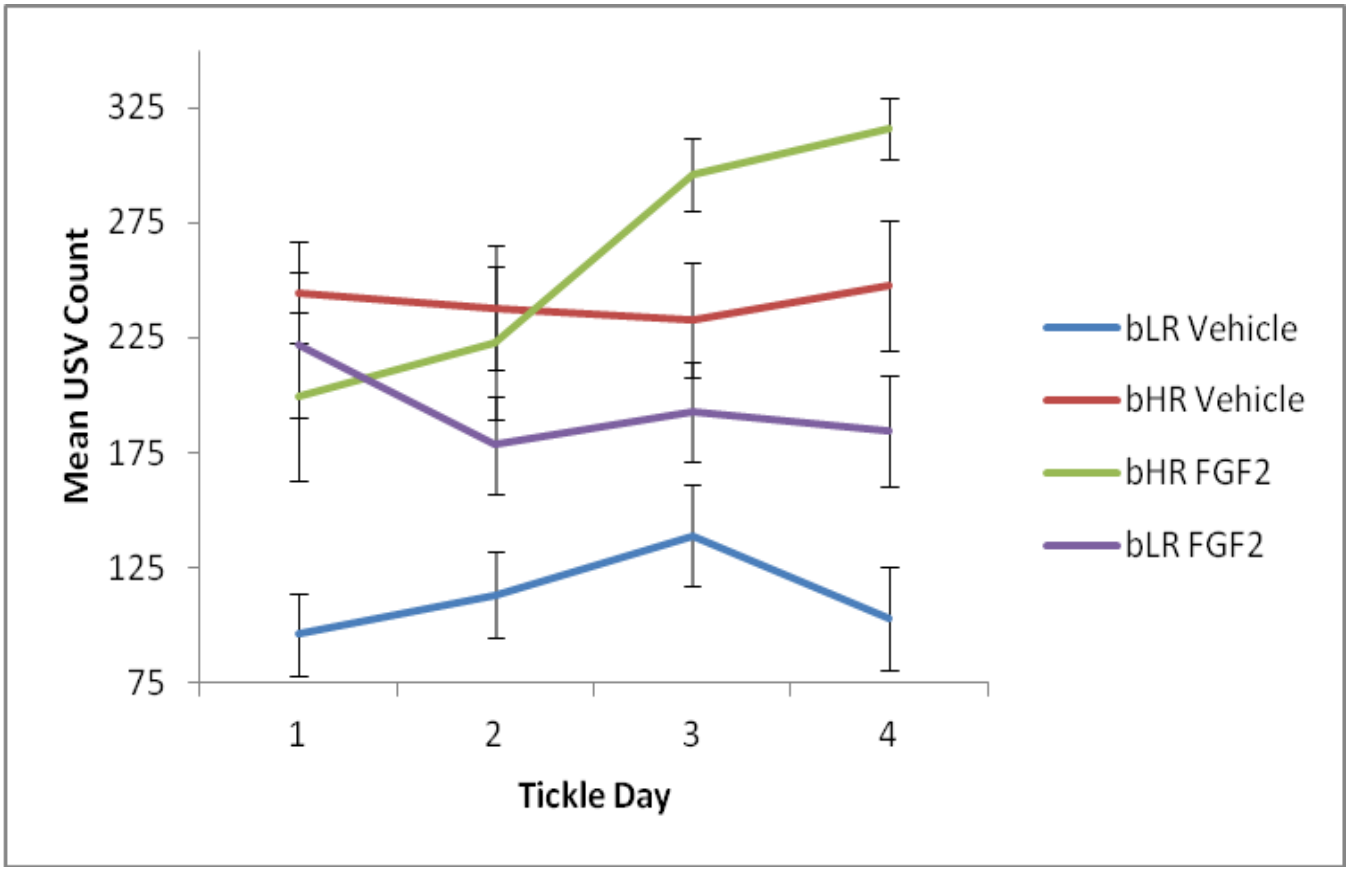

325
275
225
175
125
75
Mean USV Count
1
2
3
4
Tickle Day
bLR Vehicle
bHR Vehicle
bHR FGF2
bLR FGF2

Figure 11.

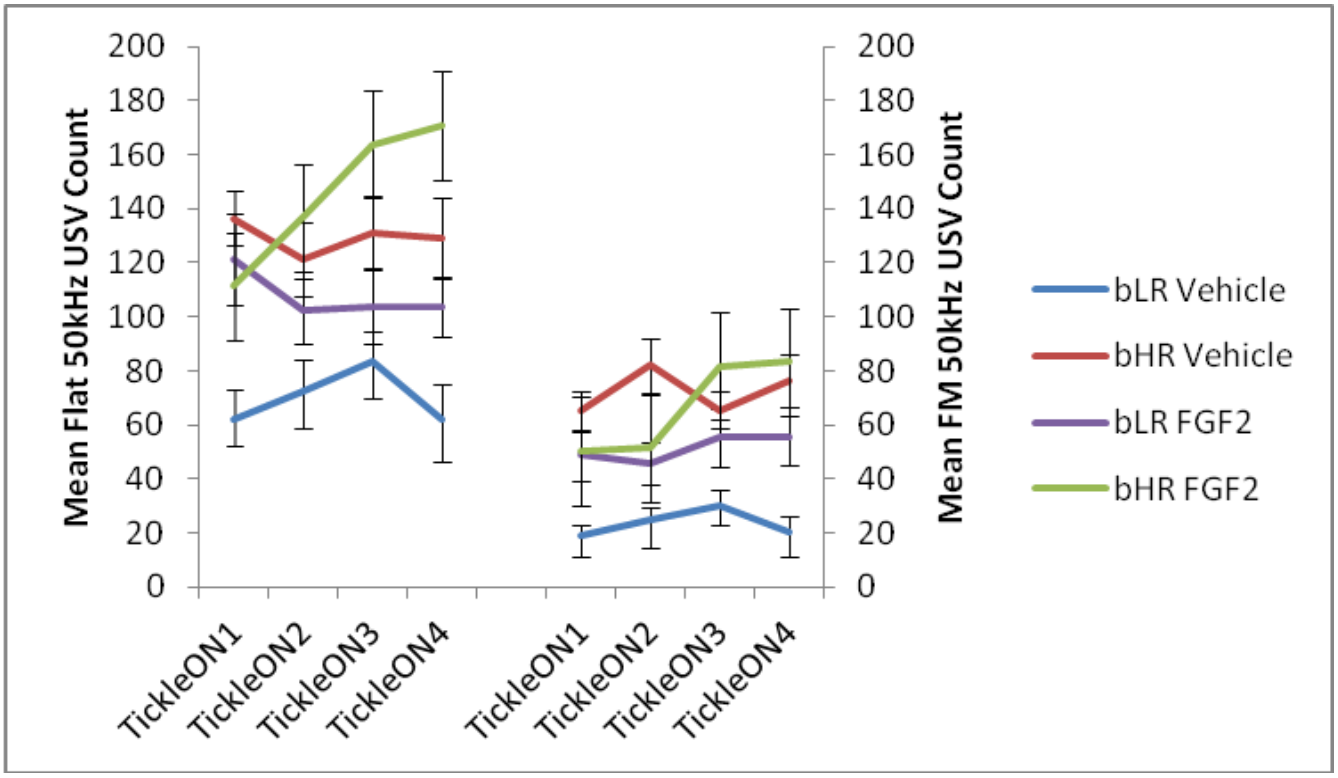

Mean Flat 50kHz USV Count
Mean FM 50kHz USV Count
200
180
160
140
120
100
80
60
40
20
0
TickleON1
TickleON2
TickleON3
TickleON4
bLR Vehicle
bHR Vehicle
bLR FGF2
bHR FGF2

Figure 12.

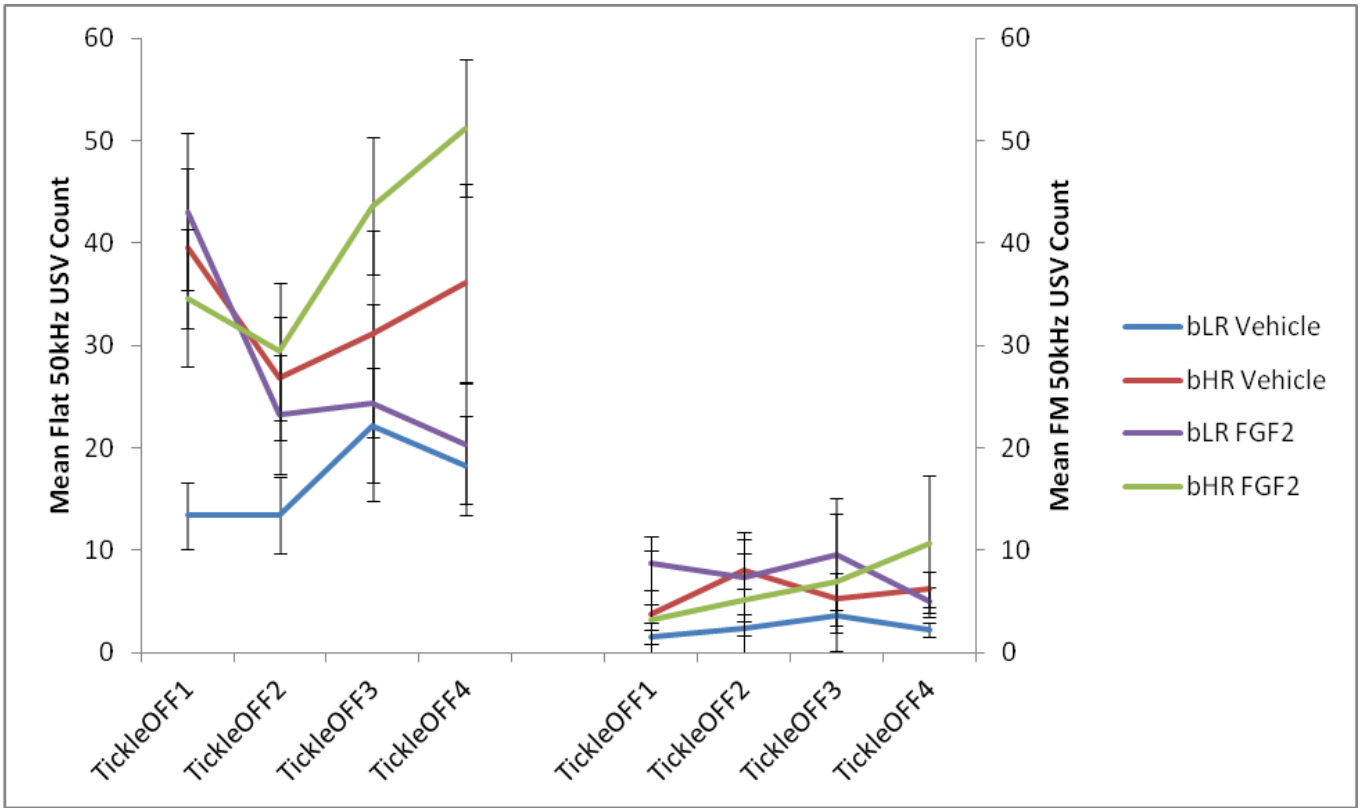

Mean Flat 50kHz USV Count
Mean FM 50kHz USV Count
60
50
40
30
20
10
0
TickleOFF1
TickleOFF2
TickleOFF3
TickleOFF4
bLR Vehicle
bHR Vehicle
bLR FGF2
bHR FGF2

Figure 13.

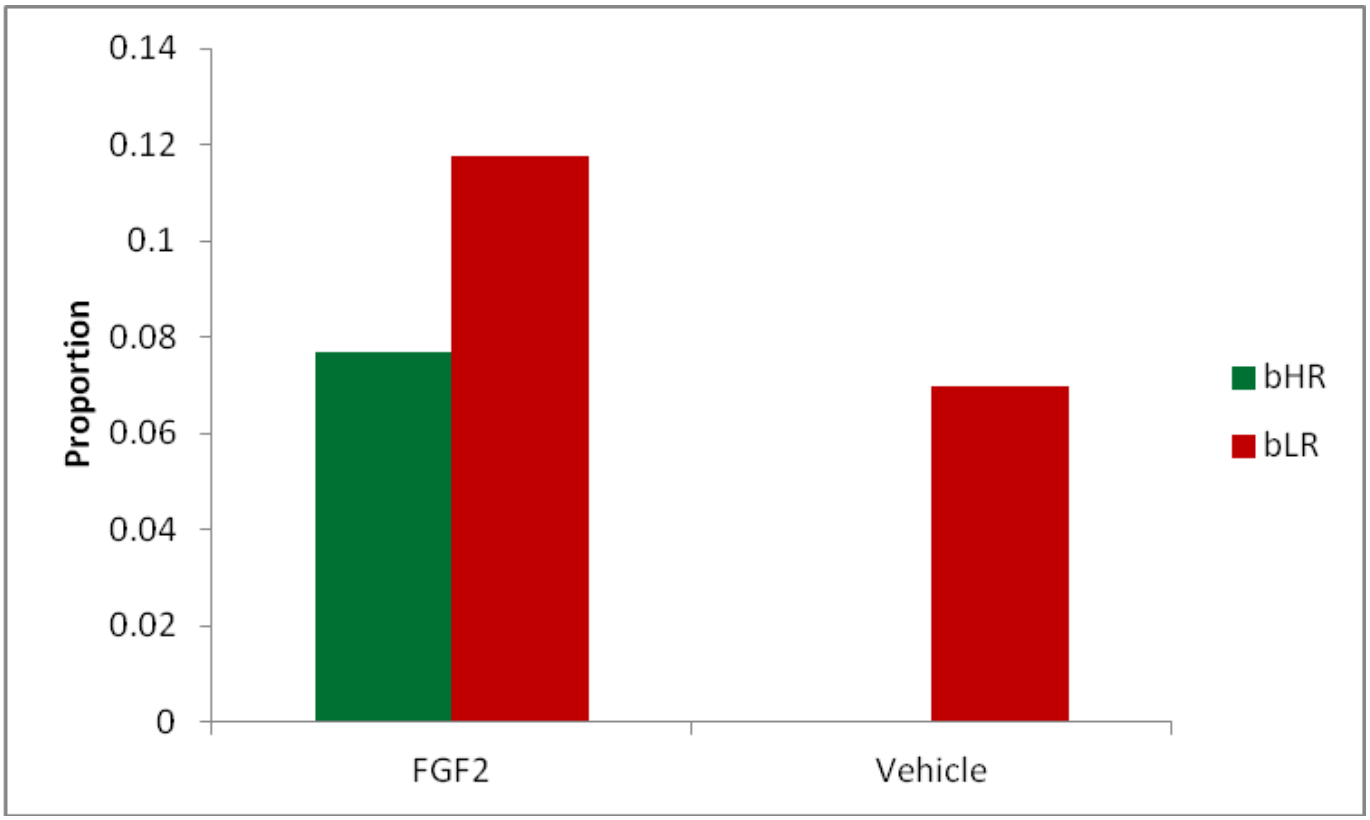


Figure 14.

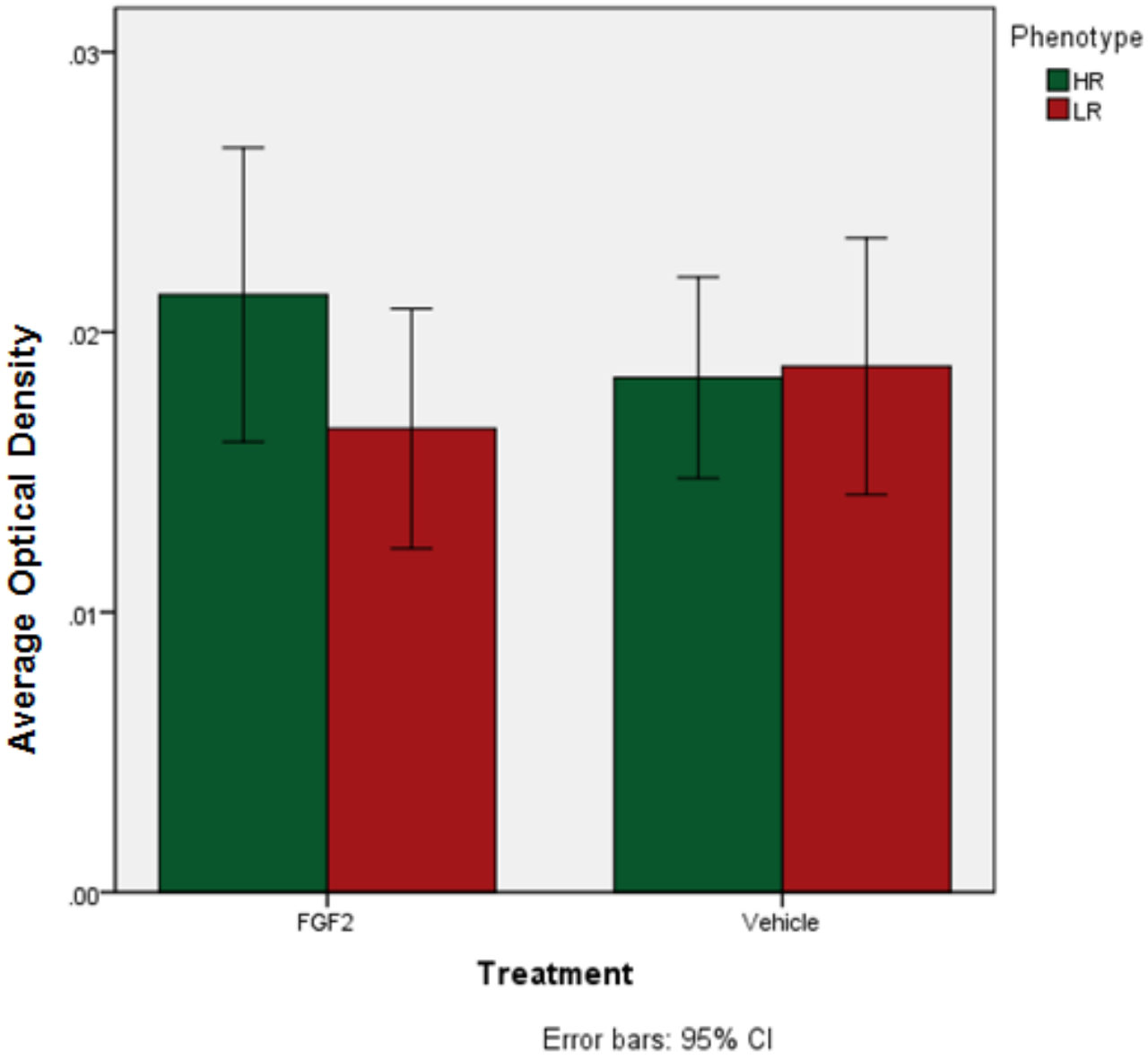
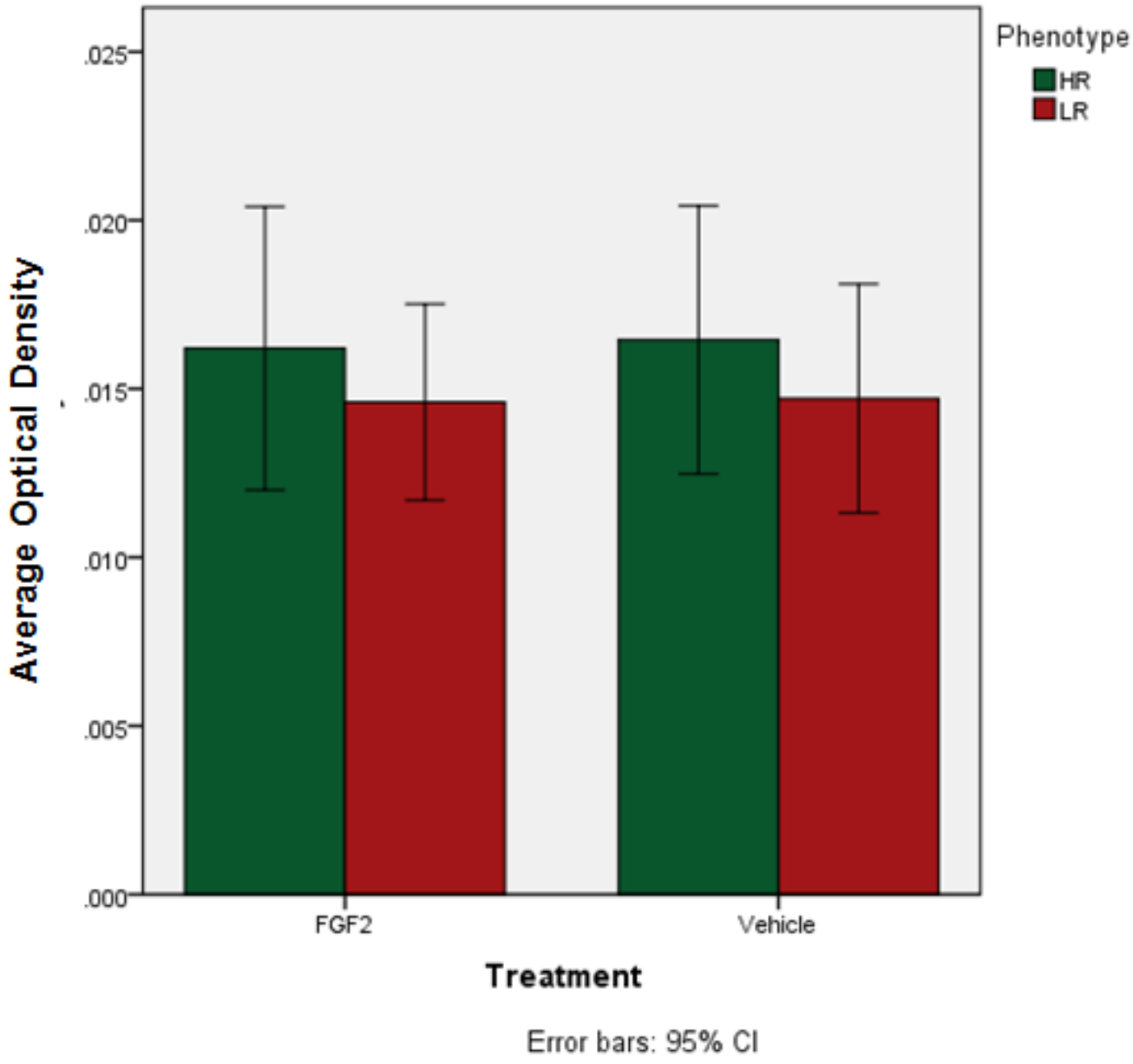

Phenotype
HR
LR
Average Optical Density
.03
.02
.01
.00
FGF2
Vehicle
Treatment
Error bars: 95% CI

Figure 15.


Phenotype
HR
LR
Average Optical Density
.025
.020
.015
.010
.005
.000
FGF2
Vehicle
Treatment
Error bars: 95% CI