Jeffrey Burgdorf[1]
Jaak Panksepp[1,2]
Stefan M. Brudzynski[3]
Margery C. Beinfeld[4]
Howard C. Cromwell[5]
Roger A. Kroes[1]
Joseph R. Moskal[1]

[1]*Department of Biomedical Engineering Falk Center for Molecular Therapeutics Northwestern University, 1801 Maple Ave. Suite 4300, Evanston, IL 60201 E-mail: j-burgdorf@northwestern.edu*

[2]*Department of Veterinary Comparative Anatomy, Physiology, and Pharmacology College of Veterinary Medicine Washington State University Pullman, WA 99163*

[3]*Department of Psychology and the Centre for Neuroscience, Brock University Saint Catharines, ON, Canada L2S 3A1*

[4]*Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA 02111*

[5]*Department of Psychology & J.P. Scott Center for Neuroscience Mind and Behavior, Bowling Green State University Bowling Green, OH 43403*

The Effects of Selective Breeding for Differential Rates of 50-kHz Ultrasonic Vocalizations on Emotional Behavior in Rats

ABSTRACT: *Fifty-kHz ultrasonic vocalizations have previously been shown to be positively correlated with reward and appetitive social behavior in rats, and to reflect a positive affective state. In this study, rats selectively bred for high and low rates of 50-kHz vocalizations as juveniles were tested as adults in a battery of behavioral tests for social/emotional behaviors. We found that animals selectively bred for high rates of 50-kHz vocalizations exhibited more crosses into the center area of the open field apparatus, were more likely to show a preference for a dilute sucrose solution (.8%) compared to tap water, and were less aggressive than randomly bred animals. Conversely, animals bred for low rates of 50-kHz calls produced more fecal boli during both open field testing and "tickling" stimulation, and made less contact with conspecifics in a social interaction test compared to randomly bred animals. We also observed that low line rats have elevated brain levels of cholecystokinin (CCK) in the cortex, which is consistent with literature showing that CCK content in the cortex is positively correlated with rates of aversive 22-kHz USVs. Conversely, high line animals had elevated levels of met-enkephalin in several brain regions, which is consistent with the role of endogenous-opioids in the generation 50-kHz USVs and positive affect. These results suggest that animals bred for high rates of 50-kHz may show a stress resilient phenotype, whereas low line rats may show a stress prone phenotype. As such these animals could provide novel insights into the neurobiology of emotion.*
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Keywords: *emotion; vocalizations; rodent*

INTRODUCTION

The study of animal vocalizations may provide insights into emotional processes (Brudzynski, Eckersdorf, & Golebiewski, 1993; Panksepp, 1981), and work along these lines in rats has focused primarily on ultrasonic vocalizations (for recent review, see Costantini & D'Amato, 2006; Portfors, 2007). In particular, 50-kHz ultrasonic vocalizations (USVs) have been hypothesized to reflect a positive affective state in rats (reviewed in Knutson, Burgdorf, & Panksepp, 2002). For example, 50-kHz USVs are elicited most robustly during the appetitive aspects of rough and tumble play and mating (Barfield & Thomas, 1986; Knutson, Burgdorf, & Panksepp, 1998; McGinnis & Vakulenko, 2003), heterospecific hand-play colloquially termed "tickling" (Panksepp & Burgdorf, 1999, 2000), following intraaccumbens injections of amphetamine (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Thompson, Leonard, & Brudzynski, 2006), and rewarding electrical brain stimulation (Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007). These pro-social vocalizations have been shown to be positively correlated with the rewarding value of rough and tumble play, social

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reunion of rats, drugs of abuse, and rewarding electrical brain stimulation (Brudzynski & Pniak, 2002; Burgdorf & Panksepp, 2006; Burgdorf et al., 2007).

To date, the data on the neurobiology and pharmacology of 50-kHz USVs is consistent with what little is known about the neurobiological basis of human positive affect (Burgdorf & Panksepp, 2006; Knutson, Burgdorf, & Panksepp, 1999). Tickling, which elicits the highest rate of 50-kHz USVs of any behavior tested, is a very effective reward in juvenile rats (Burgdorf & Panksepp, 2001; Panksepp & Burgdorf, 1999). Aversive stimuli such as lithium chloride, footshock, bright light, predatory odor, and social defeat all robustly decrease rates of 50-kHz USVs (Burgdorf, Knutson, Panksepp, & Shippenberg, 2001; Burgdorf & Panksepp, 2006). However, there are several distinct types of 50-kHz USVs, and recent data suggest that only the ''Frequency Modulated'' variety reflects appetitive behavior, reward and positive affect, and not the sonographically monotonous ''flat'' 50-kHZ calls (Burgdorf & Panksepp, 2006; Burgdorf et al., 2007).

Adult and adolescent rats also exhibit 22-kHz USVs, in response to aversive stimuli such as social defeat, predatory exposure, footshock, or other milder aversive stimuli such as an air puff (Brudzynski, Ociepa, & Bihari, 1991; Covington & Miczek, 2003; Burgdorf, Knutson, & Panksepp, 2000; Burgdorf, Knutson, Panksepp, & Shippenberg, 2001; Knutson et al., 2002). These results are consistent with the hypothesis that 22-kHz USVs reflect a negative emotional state (Brudzynski, 2007; Covington & Miczek, 2003; Knutson et al., 2002).

In a replication of and extension of earlier work (Panksepp & Burgdorf, 2000; Panksepp, Burgdorf, & Gordon, 2001), we have, for a second time, selectively bred for differential 50 kHz USVs using a tickling paradigm as the selection procedure (Burgdorf, Panksepp, Brudzynski, Kroes, & Moskal, 2005). Here we report emotional phenotyping data for animals from the 14th selection generation (S14). It should be noted that these animals were not selectively bred for the 50-kHz Frequency Modulated USVs, even though when evaluated for this parameter in the 14th generation, high line animals exhibit greater elevations of the Frequency Modulated than the flat calls as compared to controls (Fig. 3 of present study).

Previously, it has been reported that animals bred for high rates of 50-kHz calls showed both elevated rates of 50-kHz USVs as well as a concomitant decrease in negative affective 22-kHz USVs compared to randomly bred animals. Conversely, animals bred for low rates of 50-kHz USVs exhibited both lower rates of 50-kHz USVs as well elevated rates of 22-kHz USVs compared to randomly bred animals (Burgdorf et al., 2005; Harmon et al., 2008). These data suggest that high line animals exhibit a *greater* dispositional tendency for positive affectivity and a *lower* dispositional tendency for negative affectivity. Conversely, these data also suggest that low line animals exhibit a *lower* dispositional tendency for positive affectivity and a *greater* dispositional tendency for negative affectivity.

In this present study, we examined the behavioral consequences of selective breeding for high and low rates of 50-kHz USVs on a battery of emotional/motivational tasks in order to further test the hypothesis that selective breeding for 50-kHz USVs reliably reflects an underlying emotional process within the brain. Specifically, the tendencies of these three selectively bred lines (high, low, and random) to exhibit behavioral differences in an open-field test, adult ''tickle'' responsivity, gregariousness, social defeat, and Porsolt swim test were evaluated. Additionally, we examined regional brain levels of CCK and met-enkephalin which has been functionally linked to 22- and 50-kHz USVs respectively (Becker et al., in press; Burgdorf et al., 2007).

METHODS

Experiments were performed on total of 96 Long-Evans rats from the 14th generation of our selection lines that were derived from 8 progenitor litters (Exp. 1 and 2) and an outbred progenitor line of Long-Evans rats (Exp. 3) in three independent laboratories: Brock University, Ontario, Canada (Exp. 1), Northwestern University, IL (Exp. 2), and Bowling Green State University, OH (Exp. 3 and 4). All work was approved by the respective institutional animal research oversight committees.

Experiment 1: Normative Data for Tickle Responsivity in the Selected Lines

Subjects (Brock University). Fifty-four Long-Evans rats of both genders were used in this study from the 14th generation (S14) of selective breeding for differential rates of 50-kHz USVs (Burgdorf et al., 2005). Nine Long-Evans rats of both genders were used in this study from the 15th generation and were compared to 10 nonselected Long-Evans rats purchased from Harlan Sprague Dawley (Canada) which also provided the original progenitors for the selectively bred lines. For each generation, breeders were selected based on rates of tickle induced 50-kHz USVs on the last of four consecutive days of testing at 27 days of age. Animals with the highest rates of 50-kHz USVs from the high line and the lowest rates of 50-kHz USVs from the low line were paired for breeding at 3 months of age. Random line breeders were selected at random from the random line animals. There were no brother–sister mating pairs in any of the lines. All animals for the selective breeding program were weaned at 21 days of age and remained isolate-housed until pair-housing for breeding at approximately 3 months of age. In addition, the patterns of results across generation 2–14 are also reported (total $n = 761$). Additionally, 5 male and 5 female nonselectively bred Long-Evans rats were used to evaluate for

potential drift in tickle-induced USVs within the random line. Animals were weaned at 21 days of age and individually housed in 27 cm × 24 cm × 20 cm translucent polycarbonate cages with corncob dust-free bedding. Subjects were maintained on a 12:12 light dark cycle (lights on 8:00 am), and were given ad libitum access to Purina lab chow and tap water throughout the study.

Tickling studies. Animals received 5 consecutive days of 2 min/day ''tickling'' stimulation as described below, as well as previously (Burgdorf et al., 2005), starting at 24 days of age, with the results from the fourth day used for breeding selection, and an additional fifth day of testing included for high frequency recordings (only in S14). USVs were recorded and scored in a blind manner identical to the method described below. Fifty kHz USVs were categorized into ''flat'' and ''Frequency Modulated (FM)'' types as described in Burgdorf and Panksepp (2006). Fifty-kHz USVs have a bimodal distribution in bandwidth, and calls with a bandwidth less then 18-kHz are considered flat and calls with greater then 18-kHz are considered frequency modulated (Burgdorf et al., in press). Flat calls only contain the flat 50-kHz component (peak frequency ~55-kHz, non-modulated), whereas the FM calls primarily contain the flat component along with either the step or trill component (peak frequency of 35 to 70-kHz, respectively). Therefore, these compound calls (trill component alone) have greater bandwidth. Fifty-kHz USVs are defined as calls with a peak frequency greater the 35-kHz (Brudzynski, 2005). Sonograms were generated with a Flat Top window at an FFT (Fast Fourier Transform) length of 256, overlap of 50%, and a sampling frequency of 196-kHz.

Experiment 2: Open-Field, Adult Tickling Responsivity and Resident Intruder Studies in Selectively Bred Animals

Subjects (Northwestern University). Twenty-four adult male Long-Evans rats from the 14th selection generation (S14) of animals selectively bred for differential rates of 50-kHz ultrasonic vocalizations (high line, random line, and low line) were used in these studies (Burgdorf et al., 2005). Animals were weaned at 21 days of age, and remained singly housed in 20 cm × 40 cm × 20 cm translucent polycarbonate cages with corncob dust-free bedding. Animals were 3 months of age during behavioral testing. Selection occurred at 27 days of age (Panksepp & Burgdorf, 2000). Resident Long-Evans male rats ($n = 5$) used in the social defeat tests were at least 6 months old and pair housed with 3-month-old oviduct ligated females. Residents had been prescreened to exhibit consistent aggressive behaviors, and females were removed before the start of testing.

Behavioral Testing (Northwestern University)

Open field test. Animals were tested in a 40 cm × 40 cm × 20 cm high opaque Plexiglas open field cage divided into 9 equal sized 13.3 cm × 13.3 cm sections under dim lighting (~2 lux) for 10 min on two consecutive days in a testing room separate from the colony room. Sessions were recorded with a commercially available video camera and DVD recorder (Panasonic, Secaucus, NJ), and analyzed off-line in a blind manner. Line crosses, crosses into the center area, rears, and fecal boli were recorded. Boli and urine were removed from the apparatus and the chamber was cleaned with water and wiped dry with a paper towel after each test. Data from both test days were averaged for statistical analysis.

Social contact. Animals were assigned to pairs by line and body weight. One animal in the pair was placed into the home-cage of his pair-mate in the testing room for a single 30 min session under dim lighting. Sessions were recorded with a commercially available video camera and DVD recorder, and contact time (total time that animals were touching each other) was analyzed off-line in a blind manner. Given that social contact could be initiated by either member of the test pair, the use of line mates as opposed to line animal being paired with a nonselectively bred confederates ensured that contact time is reflective of only the behavior of our selectively bred (or randomly bred) animals.

Tickling studies. Heterospecific hand play (dubbed ''tickling'') consisted of vigorous whole-body playful tactile simulation that included repeated pinning of the animal in a similar manner to their natural play behavior. For all animals, the tickling was done with one hand and consisted of scaled-down rapid finger and hand movements commonly used in human tickling. However, the sequence of events generally resembles rough and tumble play of juvenile rats. The apparatus and methodology of ''tickling'' was identical to Burgdorf and Panksepp (2001) and Panksepp and Burgdorf (1999, 2000). In brief, tickling was conducted in a 45 cm × 35 cm × 20 cm opaque plastic box without bedding. Even though the tactile stimulation was rapid, brisk, and assertive, care was taken not to threaten the animals. High frequency output of a Pettersson model D980 bat detector (Pettersson Elektronik AB, Uppsala, Sweden) was recorded via a Fostex FR2 field recorder (Fostex, Boonton, NJ) and USVs were analyzed in a blind manner from sonograms generated by SASLab Pro (Avisoft Bioacoustics, Berlin, Germany) on a personal computer. In addition, fecal boli were quantified during the final day of tickling. After testing, the observation chamber was cleaned with water and paper towel if fecal boli and urine were present.

Social defeat testing. Long-Evans residents were preselected based on their propensity to initiate intermale aggression when pair housed with a tubally ligated Long-Evans female. Neither the resident animal or tubally ligated female were from the selectively bred lines. Approximately 30 min before testing, the female was removed from the male's cage. Social aggression testing consisted of placing the intruder (experimental) animal into the home-cage of the resident animal for 30 min under dim (~2 lux) light. Ultrasonic vocalizations were recorded and analyzed as described previously. Sessions were recorded with a commercially available video camera and DVD recorder. Bites and freezing behavior were recorded individually for both animals in the testing pair (i.e., resident and intruder animals). A freezing bout was counted after ~5 consecutive seconds without movement (except sniffing).

Sucrose preference. Animals were given access for 24 hr to a drinking bottle containing .8% sucrose in tap water and another

bottle containing just tap water for four consecutive days. Amount of sucrose and water consumed were recorded each day by weight, and the volume of sucrose solution consumed as a percentage of total consumption was calculated for each day. Percentage of test days in which each animal showed a preference for sucrose (more then 50% total fluid intake being from the .8% sucrose solution) were calculated.

Experiment 3: Open-Field Studies in Nonselected Rats (Bowling Green State University)

Eighteen adult female Long-Evans rats born and bred at the Bowling Green State University animal facilities, which did not originate from our behavioral selection colony, were used in this study. These animals were previously shown to have either spontaneously high ($n = 9$) or low ($n = 9$) rates of tickle induced 50-kHz calls, and were chosen from a larger group ($n = 68$) of randomly bred Long-Evans rats based on their differential tickle-induced vocalization rates. All animals were weaned at 21 days of age and singly housed in 20 cm × 40 cm × 20 cm translucent polycarbonate cages with corncob dust-free bedding. Animals were 3 months old at the start of testing.

Open Field Test. Animals were tested identically to the procedure described above except that in this present study, testing occurred under red lighting and behaviors were scored on-line by an experimenter in an experimentally blind manner.

Experiment 4: Porsolt Swim Test in Selectively Bred Animals (Bowling Green State University)

Eighteen low, 18 random, and 20 high line rats of both genders from the S14 generation were used in this study. All animals were weaned at 21 days of age and either singly ($n = 14$) or pair ($n = 44$) housed in 20 cm × 40 cm × 20 cm translucent polycarbonate cages with corncob dust-free bedding. Animals were 3–6 months old at the time of testing.

Porsolt Swim Test. A modified version of this test for adult rats, which uses a slightly larger tank (18 cm diameter × 45 cm height filled to 30 cm with 22–23°C water) to accommodate their increased size as compared to mice, was used in this study (Page, Detke, Dalvi, Kirby, & Lucki, 1999). Animals received one 15 min habituation swim session on the first day, and a 5 min tests swim session on the second day. Floating behavior (as defined by the minimal amount of movement required to keep the rat's head above water), climbing, and fecal boli were recorded by a trained experimenter in a blind manner during both the habituation and test session (Page et al., 1999).

Experiment 5: Brain Levels of CCK and Met-Enkephalin in Selectively Bred Animals (Bowling Green State University)

Nine low, 6 random, and 10 high line male rats from the S14 generation were used in this study. All animals were weaned at 21 days of age and singly housed in 20 cm × 40 cm × 20 cm translucent polycarbonate cages with corncob dust-free bedding. Animals were 3 months old at the time of sacrifice. Rats were rendered unconscious with ambient carbon dioxide, decapitated and brains were rapidly removed and placed for ~30 s in ice cold phosphate buffered saline. Initial dissection of areas was first achieved by obtaining approximately 2–3 mm coronal sections with a calibrated tissue block, followed by manual dissection of brain subregions from the brain slabs as described in Panksepp, Burgdorf, Beinfeld, Kroes, and Moskal (2007). The following brain regions were dissected: OB, olfactory bulb; FCTX, frontal neo-cortex (consisting of the frontal pole just anterior to the caudate-putamen and rostral edge of diencephalon); CTX1, anterior neo-cortex over basal ganglia; CTX2, posterior neo-cortex above the diencephalon; CTX3, Remaining Cortex; BF, all basal forebrain ventral to the CPU including nucleus accumbens; SEPT, Septum; CPU, caudate-putamen which included the septum; THAL, the entire thalamus; HYP, hypothalamus; HC, body of the hippocampus; AMY, temporal cortex ventral to rhinal fissure which included the amygdala; TECT, tectum including colliculi; TEG, tegmentum consisting of the ventral midbrain including the SN-VTA; PAG, Periaqueductal gray; BSM, lower brainstem consisting of remaining pons and medulla. All work was conducted on an ice-cold platform, using microdissection tools.

Radioimmunoassay. Biochemical procedures were essentially the same as previously described (Beinfeld, Meyer, Eskay, Jensen, & Brownstein, 1981). The brain samples were homogenized in .1 N HCL, an aliquot was taken for protein-determination (Bradford Method), and another was taken for radio-immunoassays of CCK or Met-Enkephalin. For CCK, the utilized RIA method detects mainly CCK-8, including both sulfated and unsulfated varieties, with a moderate preference for the sulfated form. It has little affinity for CCK-4 or -5, of which there is, in fact, very little in rat brain. The met enkephalin RIA utilized an antiserum directed against methionine enkephalin sulfoxide, as previously described (Kumar et al., 1990). As it is highly specific for methionine enkephalin sulfoxide, the brain samples were oxidized with .1% hydrogen peroxide before assay. The antiserum displays less than .1% cross reactivity with leucine enkephalin, dynorphin A or B, or β-endorphin (Kumar et al., 1990). Data are expressed in ng of peptide/mg of total protein for each brain sample.

Statistical Analyses

All data were analyzed with ANOVA with one factor being Line (high, random, and low). Fisher LSD post hoc tests were conducted comparing either high vs. random line or low versus random line. Data that failed Kolmogorov–Smirnov test were analyzed by nonparametric ANOVA (Kruskal–Wallis) and post hoc tests (Dunn's). For experiment 4, the false discovery rate (probability that a significant neurochemical change in a specific brain region may represents a Type II error) was calculated for each post hoc comparison by comparing the observed data versus 5,000 iterations of randomly permuted data using Excel (Microsoft, Redmond, WA), as described in Panksepp et al. (2007).

RESULTS

Experiment 1: Tickle Studies for Adolescent S2–S14 Animals

A 3 (high, random, and low line) × 13 (generations S2–S14) ANOVA was conducted followed by individual 2 × (high vs. random or low vs. random) × 13 (generations S2–S14) ANOVAs. There was a significant line effect across generations for 50-kHz USVs ($F(2, 492) = 329.9$, $p < .0001$). As depicted in Figure 1 (top graph), across 14 generations of selective breeding, high line animals exhibited higher rates of 50-kHz USVs than randomly bred animals ($F(1, 24) = 285.65$, $p < .0001$). This effect became more pronounced across generations as indicated by a Line × Generation interaction ($F(12, 24) = 4.40$, $p < .0001$). Low line animals exhibited lower rates of 50-kHz USVs compared to randomly bred rats across 14 generations of selective breeding ($F(1, 30) = 21.67$, $p < .0001$), This effect became more pronounced across generations as indicated by a Line × Generation interaction ($F(12, 30) = 3.03$, $p < .001$). A Fisher LSD post hoc analysis comparing rates of 50-kHz USVs in the S2 to the S14 generation for each line revealed that high line animals increase ($p < .0005$), low line animals decreased ($p < .0001$), and random line animals did not

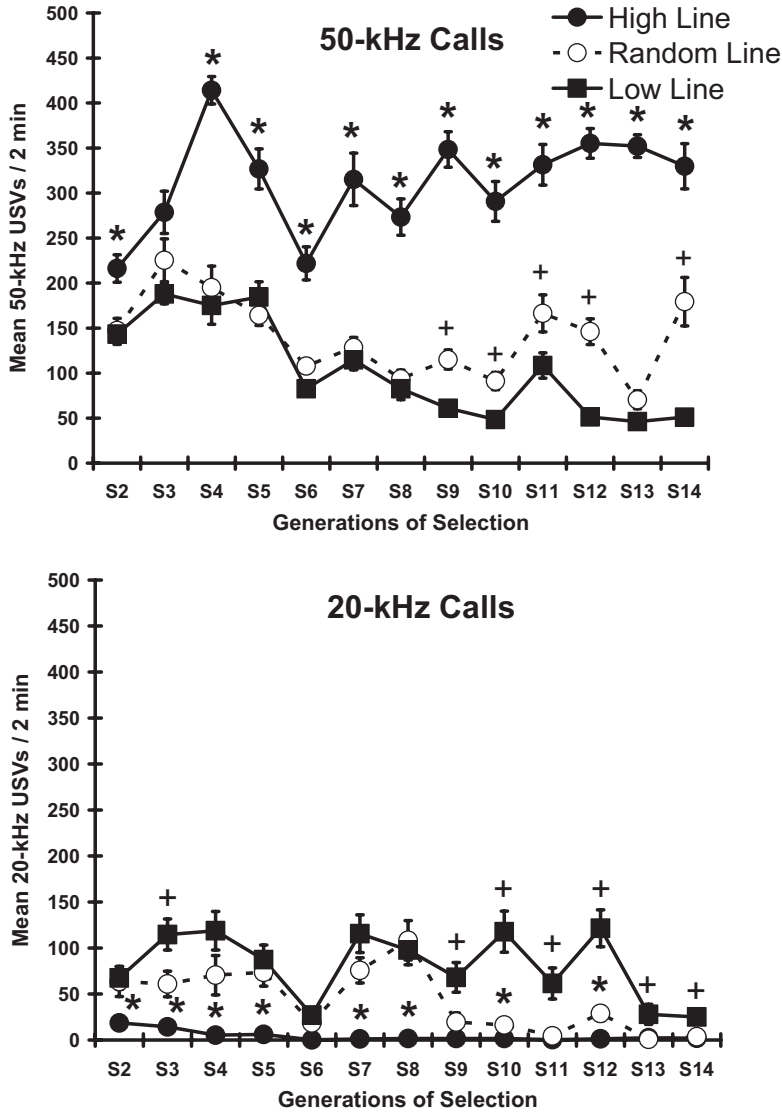

FIGURE 1 Mean ± SEM 50-kHz (top graph) and 22-kHz ultrasonic vocalizations (bottom graph) in response to two minutes of tickling stimulation at 27 days of age across 14 generations of selective bred for high, random, or low rates of tickle induced 50-kHz USVs. $^{*}p < .05$ high versus random line animals, $^{+}p < .05$ low versus random line animals, per generation (S2–S14) of selective breeding.

change ($p > .05$) rates of 50-kHz USVs comparing S2 to S14.

There was a significant line effect across generations for 22-kHz USVs ($F(2, 12) = 86.4$, $p < .0001$). As depicted in Figure 2 (bottom graph), high line animals exhibited lower rates of 22-kHz USVs as compared to random line animals ($F(1, 24) = 65.68$, $p < .0001$), and this effect became more pronounced across generations as indicated by a Line × Generation Effect ($F(12, 24) = 4.22$, $p < .0001$). Low line animals exhibited higher rates of 22-kHz USVs as compared to randomly bred animals ($F(1, 30) = 35.20$, $p < .0001$), and there was a significant line by generation effect ($F(12, 30) = 1.78$, $p < .05$). Fifty-kHz USVs were significantly negatively correlated with rates of 22-kHz USVs across all animals tested ($r = -.41, p < .0001$, Pearson correlation). Animals selectively bred for either high, low, or random rates of 50-kHz USVs did not differ in body weight on the final day of testing (postnatal day 27) when compared to each other (all p's $> .05$). No gender difference in tickle-induced USVs was evident (all p's $> .05$). No differences in rates of 50-kHz or 22-kHz USVs were found between random line animals and nonselectively bred rats ($p > .05$, data not shown).

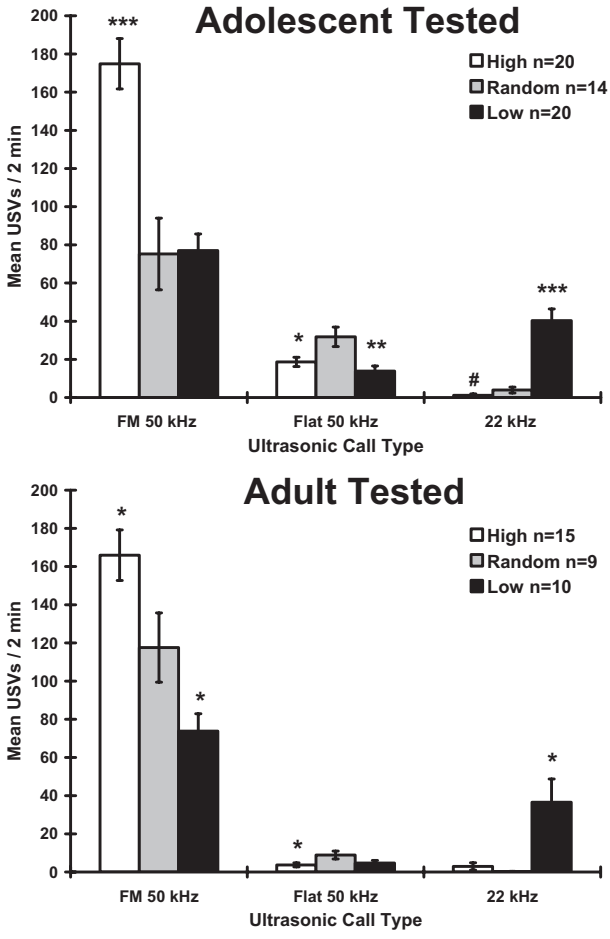

FIGURE 2 Mean ± SEM tickle-induced ultrasonic vocalizations from separate groups of S14 selectively bred rats tested either at 1 month of age at Brock University (top) or 3 months of age at Northwestern University (bottom). Frequency modulated USVs (FM 50-kHz), constant frequency USVs (Flat 50-kHz), and 22-kHz USVs are shown. $^{***}p < .0001$, $^{**}p < .01$, $^{*}p < .05$, $^{\#}p < .10$, compared to randomly bred animals.

The data were reanalyzed using a maximum of 1 animal/litter/sex to control for potential litter effects. Similar to the above analyses, a one-way ANOVA revealed a main effect for Line for both 50-kHz USVs ($F(2, 171) = 107.5$, $p < .0001$) and 22-kHz USVs ($F(2, 171) = 26.3$, $p < .0001$). Post hoc analysis revealed that high line animals exhibited more 50-kHz USVs and less 22-kHz USVs than the random line animals and that low line animals exhibited less 50-kHz USVs and more 22-kHz USVs than random line animals (all p's $< .05$).

Tickle Studies of Both Adult and Adolescent Animals. A 2 × 3 ANOVA was used to analyze this data with the factors being age (adolescent and adult) and line (high, random, and low). As depicted in Figure 2, total 50-kHz USVs were significantly altered between lines ($F(2, 31) = 27.3$, $p < .0001$), and post hoc analysis revealed that high line animals exhibited more 50-kHz USVs and the low line exhibited less 50-kHz USVs than the random line (p's $< .05$). Frequency modulated 50-kHz USVs were also significantly altered between lines ($F(2, 31) = 28.8$, $p < .0001$), and post hoc analysis revealed that high line animals exhibited more frequency modulated 50-kHz USVs than the random line ($p < .05$). Flat 50-kHz USVs were significantly altered between lines ($F(2, 31) = 17.1$, $p < .0001$), and post hoc analysis revealed that high line and low line animals exhibited fewer flat 50-kHz USVs than the random line (p's $< .05$). Twenty-two-kHz USVs were also significantly altered between lines ($F(2, 31) = 25.0$, $p < .0001$), and post hoc analysis revealed that low line animals exhibited more 22-kHz USVs than the random line ($p < .05$). Low line animals exhibited a greater number of fecal boli compared to random line animals as indexed by a main effect of line ($F(2, 31) = 17.1$, $p < .0001$) followed by a post hoc analysis between low and random line ($p < .0001$). Mean ± SEM values for fecal boli were as follows; high line ($.53 \pm .19$), random line ($.56 \pm .24$), low line ($2.20 \pm .25$).

Adult animals exhibited fewer flat 50-kHz USVs than adolescent animals irrespective of line ($F(1, 31) = 45.3$, $p < .0001$). Adult and adolescent animals from each line did not differ in rates of total 50-kHz USVs, frequency modulated 50-kHz USVs, or 22-kHz USVs (all p's $> .05$). Adolescent random line animals did not differ significantly in rates of 50- or 22-kHz calls from nonselectively bred animals (all p's $> .05$).

Experiment 2 and 3: Studies of Other Emotional and Motivational Behaviors

Open Field. As depicted in Figure 3, animals selectively bred for high rates of 50-kHz USVs exhibited more crosses in the central area of the open field than random line animals (line effect $F(2, 33) = 6.1$, $p < .01$; post hoc comparing high vs. random line, $p < .05$), and tickle-responders exhibited more crosses in the central area of the open field then tickle nonresponders ($F(1, 17) = 11.6$, $p < .005$). Low line rats exhibited more fecal boli than random line rats (line effect $F(2, 33) = 3.5$, $p < .05$; post hoc comparing low vs. random line, $p < .05$), with a nonsignificant trend in the same direction comparing tickle responders to tickle nonresponders ($F(1, 17) = 3.7$, $p < .10$). Low line rats exhibited less rearing than random line rats (line effect $F(2, 33) = 7.4$, $p < .005$; post hoc comparing low vs. random line, $p = .005$) whereas rears did not differ between tickle responders (mean $\pm$ SEM, 27.9 ± 2.9) compared to nonresponders (27.6 ± 3.1). Line crosses did not differ between tickle responders (mean $\pm$ SEM, 56.3 ± 3.6) compared to nonresponders (52.5 ± 5.8) or between the high (108.8 ± 6.9), random (95.6 ± 8.5), or low line (103.0 ± 6.9) animals. Tickle responders did not differ significantly in rates of 50- or 22-kHz USVs compared to S14 high line animals, and tickle nonresponders did not differ significantly in rates of 50- or 22-kHz USVs compared to low line animals (all p's $> .05$).

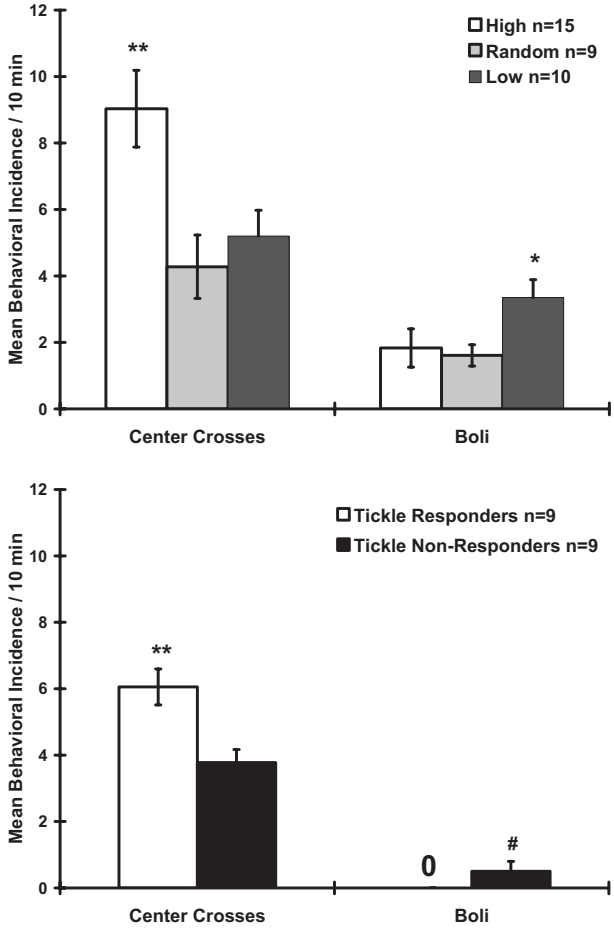

FIGURE 3 Mean $\pm$ SEM number of line crosses into the center compartment of an open field (Center Crosses) and number of fecal boli (Boli) during open field testing. Results of animals from the 14th generation of selective breeding (top graph) or randomly bred animals separated into top and lower quartile of tickle-induced 50-kHz ultrasonic vocalizations and termed tickle responders and tickle nonresponders respectively (bottom graph) are shown. $^{**}p < .01$, $^{*}p < .05$, $^{\#}p < .10$, compared to random bred animals (top graph) or tickle responder vs. nonresponder (bottom graph).

Social Behavior. Low line rats exhibited less contact time in the social contact experiment compared with the random line as revealed by a main effect across lines for contact time ($F(2, 14) = 6.6$, $p < .05$) and a post hoc test between low and random line animals ($p < .05$; Fig. 4). High line rats also exhibited fewer bites towards the resident animals in the social defeat test as indicated by a main effect for line ($F(2, 32) = 4.2$, $p < .05$; Fig. 5) and a significant post hoc comparison between random and high line rats ($p < .05$). However, animals in each line did not differ in either the number of bites received from the resident (mean $\pm$ SEM: High Line $2.6 \pm .4$, Random Line $3.2 \pm .7$, Low Line $2.7 \pm .5$), the rate of 22-kHz USVs, or submissive freezing behavior (all p's $> .05$).

Sucrose Preference. High line animals showed a greater percentage of test days with a preference for sucrose

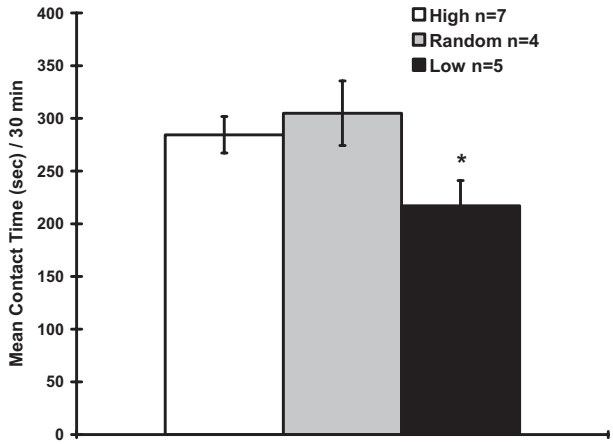

FIGURE 4 Mean $\pm$ SEM time in seconds spent in direct physical contact during a 30 min social interaction test in animals from the 14th generation tested in pairs with partners from the same selective breeding line. $^{*}p < .05$, compared to randomly bred animals.

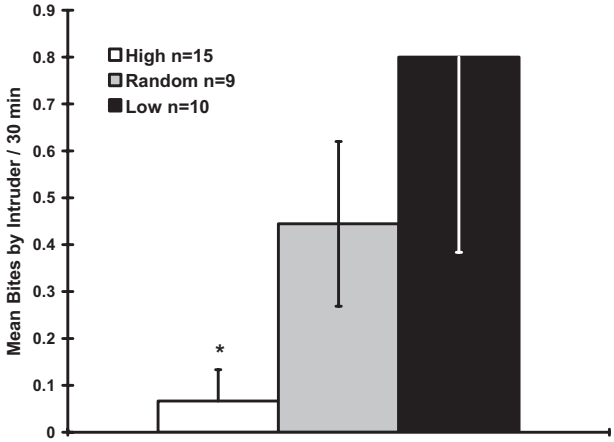

FIGURE 5 Mean ± SEM number of bites given by intruder animals from the S14 generation to nonselectively bred resident animals prescreened for high rates of aggression during the social defeat test for pairs of rats. Intruder rats regardless of line *received* on average ~2.8 bites from the resident male. $^{*}p < .05$, compared to randomly bred animals.

on average as compared to random line as indexed by a Kruskal–Wallis nonparametric one-way ANOVA (KW = 6.4, $p < .05$) followed by a Dunn's multiple comparison post hoc test comparing high versus random lines ($p < .05$). Median % of test days showing a sucrose preference was: high line (100%), random line (75%), and low line (88%). Mean ± SEM ml of sucrose intake for high/random/low line animals were: Test day 1 (25.1 ± 2.2, 23.6 ± 6.1, 27.4 ± 3.5 ml), day 2 (23.5 ± 2.7, 23.6 ± 5.8, 21.3 ± 3.6 ml), day 3 (20.1 ± 2.4, 19.2 ± 4.3, 24.3 ± 3.7 ml), and day 4 (22.5 ± 2.1, 23.3 ± 4.8, 25.5 ± 3.4 ml).

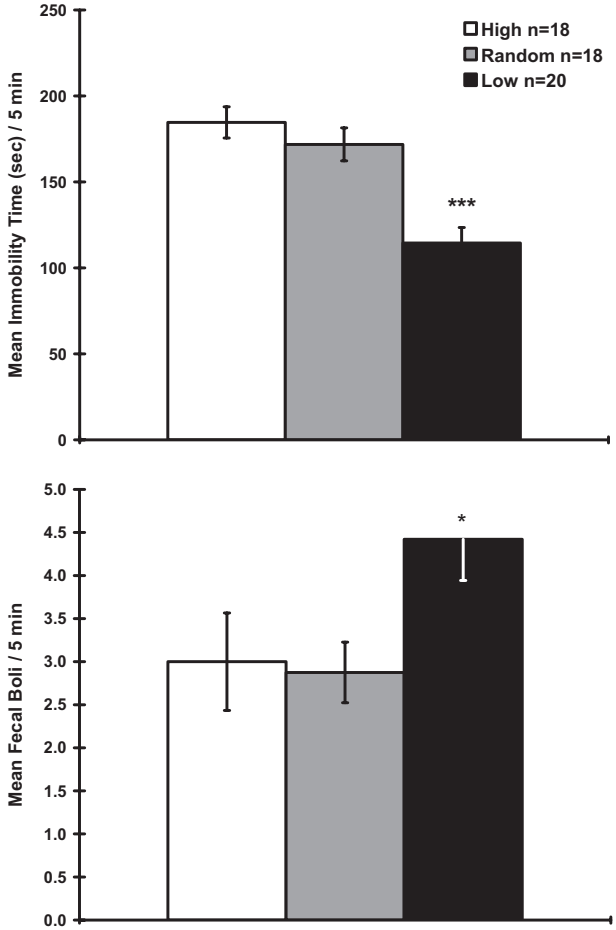

FIGURE 6 Mean ± SEM time spent floating (top panel) and number of boli (bottom panel) in the High, Random and Low line animals from the S14 generation. $^{*}p < .05$, $^{***}p < .001$ Low line compared to random line animals.

Experiment 4: Porsolt Swim Test

There was no significant effect of either gender or housing condition on either floating behavior or fecal boli (p's > .05). Therefore, the data was collapsed across gender and housing conditions for subsequent analyses. As shown in Figure 6A, low line rats exhibited less floating behavior in the Porsolt swim test on the second test day compared with the random line as revealed by a main effect across lines for floating ($F(2, 53) = 16.9$, $p < .0001$) and a post hoc test between low and random line animals ($p < .001$). Low line animals also exhibited significantly more fecal boli in the Porsolt swim on the second test day as revealed by a main effect across lines for boli ($F(2, 53) = 3.4$, $p < .05$) and a post hoc test between low and random line animals ($p < .05$; Fig. 6B). The line animals did not differ in floating time on the first day of testing (all p's > .05, data not shown).

Experiment 5: Brain Levels of CCK and Met-Enkephalin in High and Low Line Rats

As shown in Figure 7, CCK brain content varied significantly in the selectively bred lines across all brain areas assayed ($F(2, 22) = 4.7$, $p < .05$). CCK content varied significantly by brain region ($F(15, 22) = 47.0$, $p < .0001$). A significant brain region × line effect ($F(30, 22) = 3.5$, $p < .0001$) indicated that difference in CCK levels between lines varied across brain regions. Post hoc tests reveled that CCK levels averaged across all brain regions were significantly elevated in the low line compared to either the high or random line animals (all p's < .05). CCK content was decreased in the high line animals compared the random line animals and decreased in the high line animals compared to the low line in the cortex surrounding the hippocampus (cortex 2; $p < .05$). CCK content was elevated in the low line animals

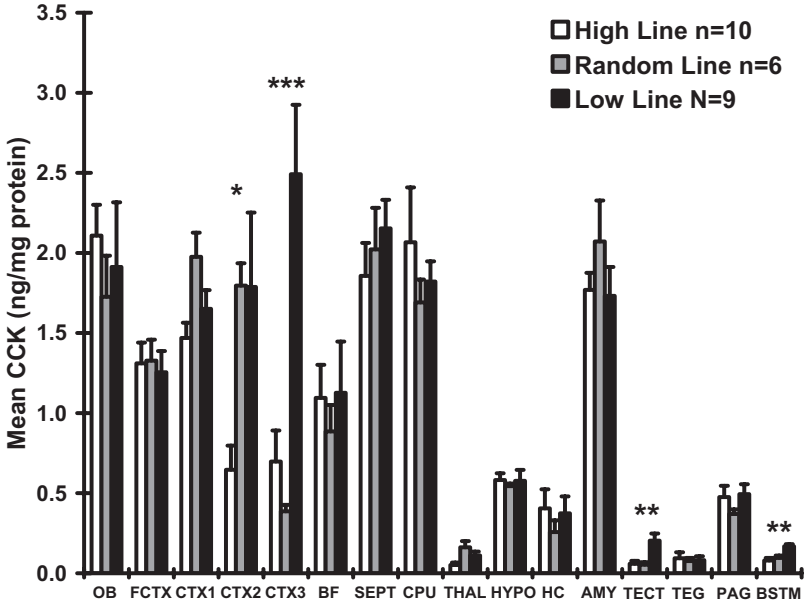

FIGURE 7 Mean ± SEM content of basal CCK-8 in tissue homogenates of various brain regions in High, Random, and Low line animals from the S14 generation. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, high or low line compared to random line animals. All significant changes had a false discovery rate of <5%.

compared the random line and elevated in the low line compared to the high line in the cortex overlying the tectum (cortex 3), the tectum proper, and the brainstem (all p's $< .05$). The false discovery rate (probability that a significant change represents a Type II error) for each of the significant changes in CCK in the above brain regions was less then 5% for each comparison.

As shown in Figure 8, Met-Enkephalin brain content varied significantly in the selectively bred lines across all brain areas assayed ($F(2, 22) = 8.3$, $p < .005$).

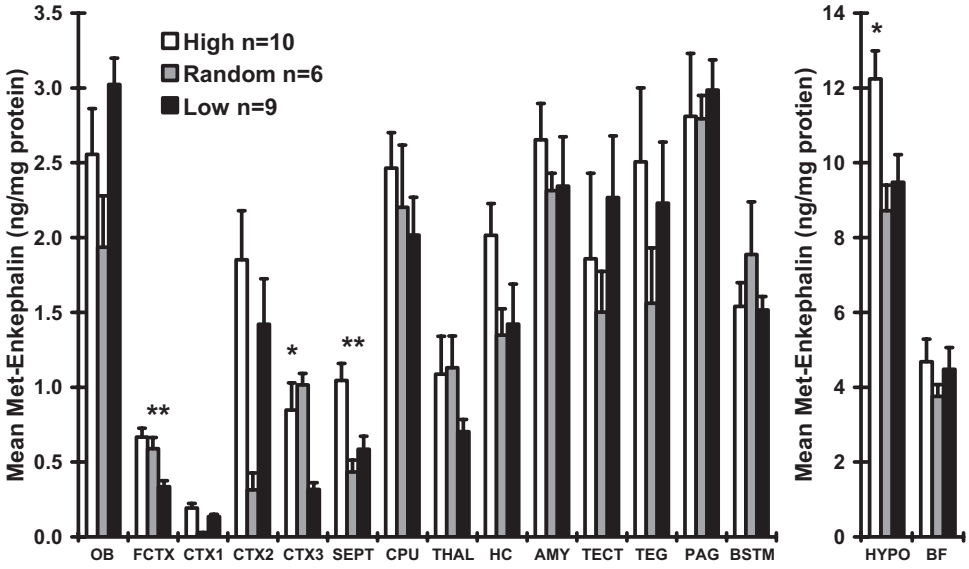

FIGURE 8 Mean ± SEM content of basal Met-enkephalin in tissue homogenates of various brain regions in High, Random, and Low line animals from the S14 generation. $^{*}p < .05$, $^{**}p < .01$, high or low line compared to random line animals. All significant changes had a false discovery rate of <5%.

Met-Enkephalin content varied significantly by brain region ($F(15, 22) = 143.0, p < .0001$). A significant brain region × line effect ($F(30, 22) = 2.5, p < .0001$) indicated that difference in Met-Enkephalin levels between lines varied across brain regions. Post hoc tests revealed that Met-Enkephalin levels average across all brain regions were significantly elevated in the high line animals compared to either the low or random line animals (all p's $< .05$). Met-Enkephalin content was increased in the high line animals compared to the random line animals and increased in the high line animals compared to the low line animals in the septum and hypothalamus (all p's $< .05$). Met-Enkephalin content was decreased in the low line animals compared the random line animals and decreased in the low line animals compared to the high line animals in the frontal cortex, and cortex surrounding tectum (cortex 3; all p's $< .05$). Met-Enkephalin content varied significantly in the above brain region and false discovery rate (probability that a significant change represents a Type II error) for each significant change was less then 1% for each comparison.

DISCUSSION

The present data support the idea that by using the selection procedures described here and previously (Burgdorf et al., 2005), we have indeed selected for differential social-emotionality. Frequency-modulated (FM) 50-kHz USVs indicate a positive emotional state (Knutson et al., 2002). The high line animals exhibited significantly more FM 50-kHz USVs compared to random line animals during tickling. In contrast, in adulthood the low line animals exhibited significantly fewer of these vocalizations than the random line animals during tickling. These results highlight the fact that Frequency Modulated USVs were more evident in animals assumed to be high in trait positive affect (high line). This agrees with previous findings that frequency modulated 50 kHz USVs are more clearly indicative of positive affect and reward than the Flat 50 kHz USVs (Burgdorf & Panksepp, 2006; Burgdorf et al., 2007). We also report that line animals showed the same vocalization patterns when tested in adulthood at 3 months of age as when tested in adolescence at 27 days of age. These results show that a stable trait has been selected.

The results of additional tests of emotionality also support the working hypothesis that high line animals have greater positive affectivity and less negative affectivity than randomly bred animals. This was based, in part, on the higher rates of positive emotional 50-kHz USVs with a concomitant decrease in the negative affective 22-kHz USVs. Animals selectively bred for high rates of 50-kHz USVs exhibited less anxiety and defensive aggression as compared to randomly bred animals in the present study, adding support to this hypothesis. High line animals also demonstrated a mild increase in preference for a dilute (.8%) sucrose solution than random line animals. In this particular study, only a preference for sucrose over water was seen in the high line animals compared to the random line animals, and not an overall increase in sucrose intake. Therefore, these data only modestly support the hypothesis that high line animals are more sensitive the rewarding effects of sucrose. Future studies are needed to determine if this increase in preference is specific to hedonic tastes.

Surprisingly, in the present study selectively bred animals did not differ in rates of 22-kHz USVs in response to social defeat. This could be the result of a ceiling effect due to the strong nature of social defeat induced 22-kHz USVs. Indeed, it has been previously shown that rats consistently show high rates of 22-kHz USVs with little between animal variability in this paradigm (Panksepp et al., 2007). Future studies could use an air puff to elicit 22-kHz USVs which produces less 22-kHz USVs with more variability between animals (Brudzynski & Holland, 2005).

A second aspect of our working hypothesis, also supported by these studies, is that low line animals should have higher levels of anxiety and lower levels of positive affectivity as compared to random controls. Animals selectively bred for low rates of 50-kHz USVs exhibited more fecal boli in the tickling, Porsolt, and open field test, suggesting heightened negative emotionality in these animals (Borelli, Nobre, Brandao, & Coimbra, 2004; Fulk et al., 2004). Low line animals also showed less social contact time compared to randomly bred animals, which has also been interpreted to reflect an anxiogenic phenotype (File, 1980). The decrease in contact time in low line animals could also be interpreted as indicative of a decreased social reward, as measured by socially induced place preferences (Panksepp, Nelson, & Bekkedal, 1997). In related work, animals selectively bred for low levels of 50-kHz USVs showed higher rates of infant separation distress calls compared to the random line animals, which further suggests that the low line animals display a high anxiety phenotype (Harmon et al., 2008).

Low line animals showed a decrease in floating time in the Porsolt swim task on the second day, and an increase in fecal boli compared to the random line animals which may reflect a high anxiety phenotype. Studies have also shown that manipulations that increase anxiety in rats also decrease floating time in the Porsolt swim test (Butler, Weiss, Stout, & Nemeroff, 1990; Martijena, Tapia, & Molina, 1996; Tezval et al., 2004; Zhang et al., in press). Thus, the decrease in floating behavior seen in low line animals may reflect a heightened state of anxiety. Indeed, low line animals show higher levels of anxiety

compared to the random line in the social interaction test (present study) and infant separation distress (Harmon et al., 2008). However, given that antidepressants also decrease rates of floating behavior in the Porsolt test (Cryan, Valentino, & Lucki, 2005) it is possible that low line animals are less prone to depression than random and high line animals.

In contrast to the present study, recently it has been shown that rats that have low levels of tickle-induced 50-kHz USVs showed no change in floating behavior on the second test day in the Porsolt test (Mällo et al., 2007). The difference between these two studies may be explained by the use of a median spilt in 50-kHz USVs differentiating high and low responders in the Mällo et al. (2007) study and the equivalent of a upper and lower quartile split between high and low animals in the present study.

In related work, low line animals failed to show a conditioned maternal preference (Harmon et al., 2008), using the procedures of Nelson and Panksepp (1996), whereas random and high line animals did show a maternal preference (Harmon et al., 2008). Therefore, it is possible that their lower social-motivation during adulthood reduces the likelihood of depressive responses (but their higher 22-kHz USVs would suggest increased susceptibility to depression).

A number of laboratories have used animals that express differential rates of ultrasonic vocalizations in tests of emotionality (Borta, Wöhr, & Schwarting, 2006; Brunelli, 2005; Mällo et al., 2007; Naito et al., 2001; Schwarting, Jegan, & Wöhr, 2007; White, Kalinichev, & Holtzman, 2007). These studies, like those reported here, show in general that animals that emit high rates of 50-kHz USVs display lower rates of anxiety, and that animals that emit high rates of 22-kHz USVs display higher rates of anxiety. While the precise nature of the affective tendencies that have been selected for will require additional research, one interpretation is that the 50 kHz USVs emitted during natural rough-and-tumble play and heterospecific hand play (''tickling'') have an ancestral resemblance to infantile human laughter which may in turn, reflect the capacity to experience social joy (Panksepp, 2007; Panksepp and Burgdorf, 2003).

Multiple replicate lines and outbred animals were not used for the selective breeding procedure reported here. Thus, low levels of genetic diversity found in inbred strains of rats such as those used here, could lead to the selection of altered genes not directly associated with vocalizations or emotion. However, it does appear that the selection procedure has in fact, selected for the appropriate emotional vocalization phenotype because: (1) We did not breed for a behavioral phenotype not found in wildtype animals: High and low 50-kHz, USV-selected animals perform similarly on tests of affect (USVs and open field) as upper and lower quartile wildtype animals; (2) Random line animals did not significantly differ in rates of 50-kHz or 22-kHz USVs as compared to age matched nonselectively bred animals from the same strain (Long-Evans; data not shown). This suggests that any reduction in genetic diversity caused by our selective breeding procedure, in itself, has not modified overall rates of USVs, our primary depended measure of affect. Also, in the current study as well as in previous studies (Harmon et al., 2008; Panksepp & Burgdorf, 2000), selectively bred animals have demonstrated the predicted changes in emotional behaviors across various tests; (3) Our selection procedure led to elevation in CCK levels in the brain of the low line animals in the posterior cortex. We have previously shown that CCK levels in the posterior cortex are elevated following social defeat and that rates of 22-kHz USVs that occur during social defeat are positively correlated ($r = .70$) with CCK content in that brain region (Panksepp, Burgdorf, Beinfeld, Kroes, & Moskal, 2004). Social Defeat has also been shown to elevate CCK in the primary motor cortex in the frontal pole (a structure that extends from our FCTX to our CTX2 sample) as measured by microdialysis, and rates of social defeat induced 22-kHz calls and submissive behavior can be reduced by administration of CCK antagonists (Becker et al., 2001). In addition, chronic social defeat that mimics a depressive state in rats leads to chronic elevation in both basal and post defeat levels of cortical CCK, which is completely blocked by chronic antidepressant treatment (Becker et al., in press); (4) Met-Enkephalin was elevated in the high line animals compared to both the random and low line animals, with the hypothalamus being one of the significant brain regions. Previously it has been shown that ventral tegmental area (part of the hypothalamus sample) injections of the μ-opiate receptor agonist DAMGO increases rates of 50-kHz USVs (Burgdorf et al., 2007), and that rates of heterospecific play-induced 50-kHz USVs are reduced in isolate-housed rats following the μ-opiate receptor antagonist naloxone (Burgdorf & Panksepp, 2001).

Taken together, these studies suggest that conversely, animals selectively bred for high rates of 50-kHz USVs reflect a positive affective phenotype that is comparatively resistant to negative affective challenges. Whereas animals selectively bred for low rates of 50-kHz USVs exhibit behavioral changes consistent with a high negative affective phenotype. Thus, these animals may be useful in evaluating the biochemical and molecular biological changes in brain regions shown to regulate positive and negative affect (e.g., Mulligan et al., 2006).

NOTES

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