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Breeding for 50-kHz Positive Affective Vocalization in Rats

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Adolescent and adult rats exhibit at least two distinct ultrasonic vocalizations that reflect distinct emotional states. Rats exhibit 22-kHz calls during social defeat, drug withdrawal, as well as in anticipation of aversive events. In contrast, 50-kHz calls are exhibited in high rates during play behavior, mating, as well as in anticipation of rewarding events. The neurochemistry of 22-kHz and 50-kHz calls closely matches that of negative and positive emotional systems in humans, respectively. The aim of this study was to replicate and further evaluate selective breeding for 50-kHz vocalization, in preparation for the analysis of the genetic underpinnings of the 50-kHz ultrasonic vocalization (USV). Isolate housed adolescent rats (23–26 days old) received experimenter administered tactile stimulation (dubbed ''tickling''), which mimics rat rough-and-tumble play behavior. This stimulation has previously been shown to elicit high levels of 50-kHz USVs and to be highly rewarding in isolate-housed animals. Each tickling session consisted of four cycles of 15 seconds stimulation followed by 15 seconds no stimulation for a total of 2 minutes, and was repeated once per day across four successive days. Rats were then selected for either High or Low levels of sonographically verified 50-kHz USVs in response to the stimulation, and a randomly selected line served as a control (Random group). Animals emitted both 22-kHz and 50-kHz types of calls. After five generations, animals in the High Line exhibited significantly more 50-kHz and fewer 22-kHz USVs than animals in the Random Line. Animals selected for low levels of 50-kHz calls showed marginally more 22-kHz USVs then randomly selected animals but did not differ in the rate of 50-kHz calls. These results extend our previous findings that laboratory rats could be bred for differential rates of sonographically verified 50-kHz USVs.

KEY WORDS: Emotion; motivation; rats; reward; selective breeding; ultrasonic vocalization.

INTRODUCTION

Complex traits including emotional responsiveness can be readily bred for in rodents using selective breeding procedures. Roman high and low avoidance rats exhibit differential emotional reactivity to stressors (Steimer *et al.*, 2001). Wistar–Kyoto rats also exhibit an anxiogenic phenotype (Gentsch *et al.*, 1988). More recently, rats selected for high levels of pup separation distress vocalizations have been shown to exhibit an anxiogenic phenotype in adult tests of anxiety (Brunelli, in press; Dichter *et al.*, 1996; Hofer *et al.*, 2001). In mice, such calls have been shown to exhibit complex patterns of genetic and developmental influences (Thornton *et al.*, in press).

Although negative emotionality has been well studied using behavioral genetic procedures, positive emotionality has been largely neglected. This is

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despite the fact the positive emotionality has a strong genetic component in humans (Lykken and Tellegen, 1996), and positive emotional phenotype can be selected for in dogs (Scott and Fuller, 1965). Indeed, it is thought that a major aspect of domestication of species involved selecting for positive emotionality characteristic of youthful play. For example, adult dogs are more capable of engaging in play behavior then adult wolves (Scott and Fuller, 1965). The experimental analysis of play, behavior suggests strong neurobiological control of such dispositions (Panksepp *et al*., 1984; Vanderschuren *et al*., 1997). One of the most objective ways to study social processes, and potentially to select for distinct socio-emotional tendencies, is through the study of ultrasonic vocalizations (USVs) which are exhibited in a large variety of social situations (Brudzynski, in press; Hahn and Lavooy, in press).

In laboratory rats, positive affective states such as ludic playfulness or social joyfulness can be studied using measurements of 50-kHz USVs (Knutson *et al*., 2002). Adult as well as adolescent rats emit these vocalizations in response to rewarding stimuli such as food, sexual partners, play, drugs of abuse, lateral hypothalamus electrical stimulation, or social contacts after a mild isolation (Brudzynski and Pniak, 2002; Panksepp *et al*., 2002b). To the contrary, aversive stimuli such as bright light and predatory odors reduce 50-kHz vocalizations (Panksepp *et al*., 2002b).

In order to reliably induce 50-kHz USVs in juvenile rats, daily sessions of experimenter administered tickle-like stimulation (heterospecific ''play'') were administered, which mimicked, in part, tactile stimulation during the natural play behavior in rats (Burgdorf and Panksepp, 2001; Panksepp and Burgdorf, 1999). This stimulation was dubbed ''tickling'' and it gradually induced the highest levels of 50-kHz USVs of all the paradigms that elicit these USVs. Rats that exhibit higher levels of ''tickle'' induced 50-kHz USVs are more playful, and are more preferred as a play partner by a conspecific, than animals who exhibit lower levels of 50-kHz USVs (Panksepp *et al*., 2002a). Levels of ''tickle'' induced 50-kHz vocalizations are highly positively correlated with the rewarding value of the ''tickle-like'' tactile stimulation (Burgdorf and Panksepp, 2001). High levels of 50-kHz ''tickle'' induced USVs can also be facilitated using selective breeding procedures (Panksepp and Burgdorf, 1999, 2000). Animals selected for high levels of 50-kHz ''tickle'' induced vocalizations also exhibit more play behavior with conspecifics, and find this tactile stimulation to be more rewarding and less aversive than in the wild type animals (Panksepp and Burgdorf, 2000). Assuming deep homologies exist in the mammalian brain emotional systems (Panksepp, 1998; Panksepp *et al*., 2002b), a strong case can be made that this response may have an ancestral relationship to brain processes that regulate childhood laughter in humans which are play rather than humor induced (Panksepp and Burgdorf, 2003).

In contrast, adult and adolescent rats exhibit a second type of USV in the 20–30 kHz range that reflects a negative affective state (Brudzynski, 2001; Covington and Miczek, 2003; Knutson *et al*., 2002). These USVs are exhibited during social defeat, in response to danger, threat, aversive drugs, and in anticipation of foot shock (Brudzynski, 2001, 2002; Knutson *et al*., 2002). In the case of aversive drug conditioning, levels of 22-kHz USVs positively correlate with the drugs ability to produce a conditioned aversion to the drug-paired environment (Burgdorf *et al*., 2001).

Since our previous 50-kHz USV selection lines (Panksepp and Burgdorf, 1999, 2000) died unfortunately in anticipation of pursuing molecular biological work on such animals (Panksepp *et al*., 2002c), in this study, we attempted to replicate our selective breeding for high as well as low levels of ''tickle'' induced 50-kHz USVs in adolescent rats with a particular attention to the acoustic analysis of the emitted USVs. The auditory detection of the 50 kHz type of USVs within a selected frequency band in previous studies was replaced by a detailed sonographic analysis of all emitted USVs in the present study. The replication of our previous work with 50 kHz USVs with sonographically verified sound analysis not only speaks to the genetic robustness of this phenomenon but sets the stage for genetic work to track down the allelic variations that may be critical for the amplification of this emotional response.

METHODS

Animals and Housing

Male and female adolescents of the Long Evans rat strain, born and reared at the Brock University Animals Facility (St. Catherine, Ontario, Canada) were used in this study and tested at 24–26 days of age. Pregnant female rats were purchased from

Charles River (St. Constant, PQ, Canada), with their offspring (S0) being the first to receive testing. After weaning at 21 days, subjects were individually housed in $26.7 \times 24.1 \times 20.3$ translucent polycarbonate cages with corncob dust-free bedding. All animals had ad lib. access to food and water, and were maintained in constant room temperature on a 12:12 light:dark cycle with lights on at 8:00 am. All animals were studied under veterinarian supervision and individuals with a doubtful health condition were rejected from the study during culling.

Behavioral Testing

Testing was similar to that used in the previous breeding study (Panksepp and Burgdorf, 1999, 2000) and consisted of placing the subjects in a $45 \times 35 \times 20$ cm opaque plastic test box with corncob bedding. Subjects then received 2 minutes exposure to a standard tickle test-sessions consisting of four successive alternating cycles of 15 seconds of no stimulation followed by 15 seconds of "tickle" stimulation. This procedure was conducted once per day for four consecutive days. For all animals in all tests the "tickling" stimulation was performed with the right hand and consisted of rapid initial finger movements across the back of the rat with a focus on the neck, followed by rapidly turning the animals over on their backs, with vigorous alternating finger movements on their ventral surface, followed by release after a 1–2 seconds of stimulation. This procedure was termed "tickling" and was repeated throughout each session ("tickling" session). Even though, the tickling was brisk and assertive, care was taken not to frighten the animals.

Animals received four consecutive days of tickling sessions starting at 24 days of age. USVs were recorded via a bat detector (Model S-200, Ultra Sound Advice, London, England) using a 1:16 frequency division, with a condenser microphone (SM-1) placed ~35 cm above the cage floor. An attempt was made to always position the stimulated animal in the same relation to the microphone. Divided bat detector output was recorded onto standard low noise audiocassette tapes (TDK, SA-60 tape and VSC-2001 (Intertan Canada, Ltd., Barie, ON) tape recorder) for subsequent sonographic analysis. Analyses were performed on the DSP Sona-Graph digital sound processing work station (model 5500, Key-Elemetrics Corp., Pine Brook, NJ). All recorded tapes were viewed on the sonograph and the 50-kHz USVs were identified. The number of USVs emitted in the alternating 15 seconds stimulation/no stimulation bouts were counted directly from the sonograph's monitor.

Breeding Selection

Five generations of animals from the original parent generation were selectively bred using cross breeding within the respective litters of the various lines (i.e., no brother:sister matings were used, so as to minimize inbreeding fertility problems we encountered in the low-lines of our original selection program) from six different litters selected for differential 50-kHz vocalization rates. Each breeding pair sired only one litter, for a total of 2 litters/line/generation (total 6 litters per generation). 50-kHz call rates on the final test day (day 4) were used for selection. A High Line (2 litters) was selected for high 50-kHz vocalization rates, a Low Line (2 litters) was selected for low 50-kHz vocalization rates, and the Random Line (2 litters) consisted of arbitrary selected breeding pairs that remained after the extreme of low ($n = 1$) and high ($n = 1$) animals had been discounted out of a total of 20 animals for the random line. Each litter was culled to 10 pups (5 males, 5 females), thus there were 60 pups in each generation (20 in each line). After the tests were finished, the next breeders were selected and kept in isolate housing until maturity and mating time. In sum, rather than having duplicate lines within each selection group, we derived a replicate of a single line, with the plan that once we had a robust initial separation (e.g., around 8–10 generations), we would farm out lines to various distinct locations for continuation of the breeding program, which from that point on would constitute duplicate, triplicate and perhaps quadruplicate lines eventually. Anyone who is interested in having breeding stock for such lines, under a collaborative arrangement, may contact the second author.

RESULTS

S1 generation was excluded from the analysis given that lines from this generation did not consist of two breeding pairs per line, and subsequently breeding pairs where chosen that combined the selection lines with the "wild" type line. Sonographic analyses revealed that our juvenile rats generally emitted two types of USVs: long 22-kHz type and short 50-kHz type of USVs. The 22-kHz USVs were counted separately from the 50-kHz ones. Given that

for the S1 generation only, 50-kHz USVs were scored, data from those animals were not included in the analysis. As depicted in Figure 1a, levels of 50-kHz USVs increased in the animals selected for high levels of 50-kHz call compared to randomly selected wild type animals as indicated by a significant Line *versus* Generation interaction. ($F(2,52) = 63.08$, $p < 0.0001$). *Post hoc* testing revealed that the high line was significantly different from the random line ($p < 0.0001$), but the low line was not significantly different from the random line ($p > 0.05$). Levels of 50-kHz USVs also increased across generations in the high line animals more then the random line as indicated by a significant Line X Generation interaction ($F(2,6) = 6.69$, $p < 0.0001$). As depicted in Figure 1b, Levels of 22-kHz USVs were lower in high line and higher in low line animals as compared to the random line as indicated by a main effect for line ($F(2,52) = 28.23$, $p < 0.0001$). Post hoc testing revealed that the high line showed lower levels of 22-kHz USVs ($p < 0.0001$) and low line animals showed higher levels of 22-kHz USVs ($p < 0.05$) as compared to the random line. 50-kHz:USVs were significantly negatively correlated with 22-kHz call rats (Spearman's $r = -0.59$, $p < 0.0001$). There were no significant gender differences in either rate of 50-kHz or 22-kHz USVs (all p's > 0.05).

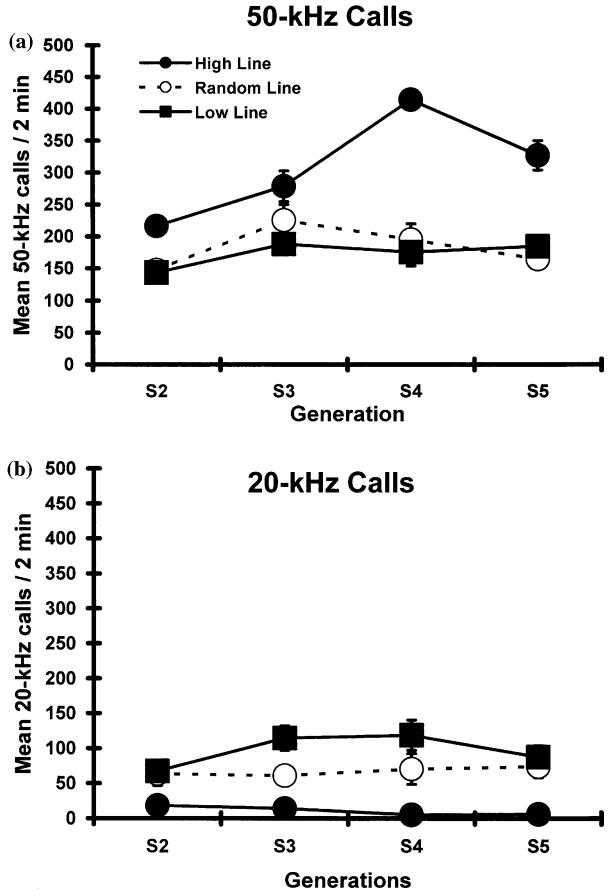

Fig. 1. Mean (±SEM) (a) 50-kHz ultrasonic and (b) 22-kHz USVs in response to 2 minutes of "tickling" stimulation in animals selectively bred for High and Low levels of 50-kHz USVs across four successive generations of selection (S2–S5). Results for the S1 generation are not provided since only 50-kHz USVs were monitored during the initial cycle.

DISCUSSION

These results replicate our previous finding that high levels of 50-kHz USVs can be experimentally bred within four generations in Long Evans rats (Panksepp and Burgdorf, 1999, 2000). Those original lines died off as a consequence of a laboratory shut-down for asbestos abatement and our inability to achieve a cross-fostering transfer of those lines to another facility, necessitated by our adults harboring a skin parasite that preclude the direct re-housing of our breeding stock.

Thus, the present results are a totally independent replicate, and a few interesting differences have become evident. Despite our ability to successfully elevate the levels of 50-kHz USVs in the High Line to about the levels obtained in our previous breeding study (Panksepp and Burgdorf, 2000), the Low Line has not yet exhibited a reduced level of 50-kHz USVs compared to the Random Line. However, the Low Line animals have exhibited slightly more 22-kHz USVs then the random or high line. This could be due to the fact that in early generations in the present study of low line animals, we may have inadvertently selected for high levels of 22-kHz USVs as well as low levels of 50-kHz USVs, whereas in the previous study, we selected for only low levels of 50-kHz USVs. In the previous study, we did not monitor the 22-kHz USVs. It is possible that in our previous study, animals selected for low levels of 50-kHz USVs reflected a relatively mute phenotype. We can be confident that this was not the case in the present study.

In any event, the failure of the Low Line to separate may not be troublesome for our eventual goal of identifying the neurogenetic substrates of the 50-kHz USVs, which was postulated to express positive affect vocalization in juvenile and adult rats (Brudzynski and Pniak, 2002; Knutson *et al.*, 2002; Panksepp *et al.*, 2002b). If our Low Line does eventually

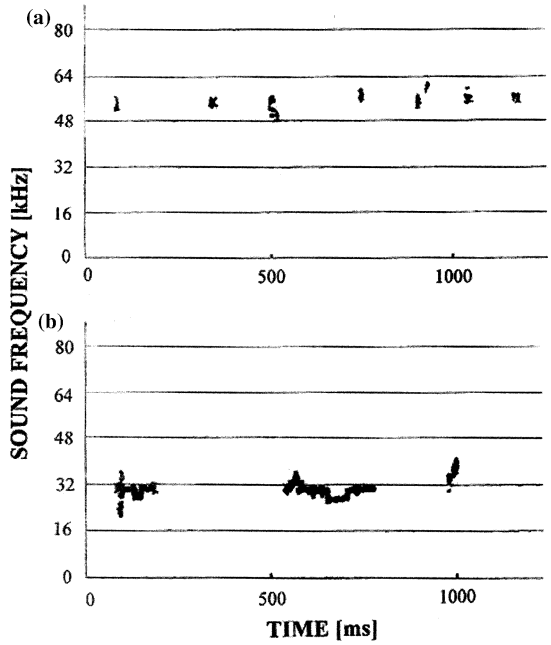

Fig. 2. Representative sonograms of "tickle" induced (a) 50-kHz ultrasonic calls and (b) 22-kHz calls in juvenile rats. Note: 22-kHz calls are shorter in duration then seen during aggressive behavior in adult animals.

separate well on the 22-kHz vocalization, we may have a potential high negative affect line which could be especially useful for genetic comparisons. Of course, these phenotypes will need to be fully evaluated using various behavioral procedures. In our previous study, only animals bred for high levels of 50-kHz USVs were significantly elevated compared to randomly selected wild type animals with respect to the rewarding value of the "tickling" stimulation and elevation of playfulness. Finally, our present observation that 50-kHz and 22-kHz call rates are negatively correlated, adds further support to the idea that these vocalizations represent an affectively distinct polarity of emotional states in rats (Brudzynski and Pniak, 2002; Budzynski, 2001; Knutson *et al.*, 2002). Previously we have found that aversive stimuli that increased levels of 20-kHz USVs (e.g., LiCL, social defeat) did so with a concomitant decrease in 50-kHz USVs (Burgdorf *et al.*, 2001; Panksepp and Burgdorf, 2003).

In agreement with this explanation, the tickling-type of heterospecific play stimulation does not unequivocally represents a "pleasurable" situation for rats at the first session. However, the decreasing rates of 22-kHz USVs and increasing rates of 50-kHz USVs in successive sessions of stimulation indicated a fast pace of learning and a presumably gradual extinction or habituation of negative affect and potentiation of the positive affective assessment of this situation. In the future work, it will be important to determine if animals selectively breed for high levels of 50-kHz USVs find human-applied tickling stimulation to be more rewarding, and whether such animals exhibit more conspecific play behavior as compared to controls. Both trends were evident in the previous set of animals (Panksepp and Burgdorf, 2000). The use of a large number of affect measures will be essential to have confidence that we do have positive and negative affect phenotypes. In any event, the aim of the future work is to be able to utilize modem molecular biological techniques to evaluate the neurochemical consequences of our selection procedure (e.g., as described in Panksepp *et al.*, 2002c).

It is important to note that this project was initiated with the specific aim of facilitating an understanding of the basic neurobiological processes that mediate a positive affect that may be characterized as social joy for both the juvenile and adult rats. We have the working hypothesis that there may be an ancestral relationship to the playful laughter of the very young members of our own species (Panksepp and Burgdorf, 2003). We know so little about human laughter (despite Provine's (2000) seminal efforts), that it is hard to empirically evaluate phylogenetic and functional homologies at the present time. An eventual comparative analysis of allelic, variations in relevant species may provide such evidence.

At present, we provisionally regard this model as a potentially fruitful one for working out some underlying psychobiological principles that may be relevant for understanding playful joy in our own species. Indeed, we have already demonstrated that placement of psychostimulants such as amphetamine into the ventral striatum (nucleus accumbens) can promote vigorous 50-kHz USVs in rats (Brudzynski, 2002; Burgdorf *et al.*, 1999). Thus, we find it most intriguing, and promising, that a recent human brain imaging study reports that extremely funny jokes provoke robust arousal of this same brain area in humans (Mobbs *et al.*, 2003). Although 50-kHz USVs in rodents may have little to do with human *humor*, our working hypothesis is that it may have physiological homology to the laughter that human boys and girls exhibit so abundantly during the human equivalent of the rough-and-tumble play in rats (Scott and

Panksepp, 2003). But even if that relationship turns out to be conceptually flawed, an understanding of the genetics of this response may provide an inroad to the systematic study of an experimentally tractable positive emotional response in another mammalian species.

REFERENCES

Brudzynski, S. M. (2001). Pharmacological and behavioral characteristics of 22 kHz alarm calls in rats. *Neurosci. Biobehav. Rev.* **25**:611–617.

Brudzynski, S. M. (2002). Fifty-kHz calls induced by direct activation of the dopaminergic system in the rat brain. *Abst. Intern. Behav. Neurosci. Soc.* **11**:70.

Brudzynski, S. M. (2005). Quantitative parameters of ultrasonic communication in rats. *Behav. Genet.* **35:**85–92.

Brudzynski, S. M., and Pniak, A. (2002). Social contacts and production of 50-kHz short ultrasonic calls in adult rats. *J. Comp. Psychol.* **116**:73–82.

Brunelli, S. A. (2005). Selective breeding for an infant phenotype: Rat pup ultrasonic vocalization (USV). *Behav. Genet.* **35:**53–65.

Burgdorf, J., Knutson, B., Panksepp, J., and Ikemoto, S. (2001). Nucleus accumbens amphetamine microinjections unconditionally elicit 50 kHz ultrasonic vocalizations in rats. *Behav. Neurosci.* **115**:940–944.

Burgdorf, J., Knutson, B., Panksepp, J., and Shippenberg, T. S. (2001). Evaluation of rat ultrasonic vocalizations as predictors of the conditioned aversive effects of drugs. *Psychopharmacol.* **115**:35–42.

Burgdorf, J., and Panksepp, J. (2001). Tickling induces reward in adolescent rats. *Physiol. Behav.* **72**:167–73.

Covington, H. E. III, and Miczek, K. A. (2003). Vocalizations during withdrawal from opiates and cocaine: possible expressions of affective distress. *Eur. J. Pharmacol.* **467**:1–13.

Dichter, G. S., Brunelli, S. A., and Hofer, M. A. (1996). Elevated plus-maze behavior in adult-offspring of selectively bred rats. *Physiol. Behav.* **60**:299–304.

Gentsch, C., Lichtsteiner, M., and Feer, H. (1988). Genetic and environmental influences on behavioral and neurochemical aspects of emotionality in rats. *Experientia* **44**:482–90.

Hahn, M. E., and Lavooy, M. J. (2005). A review of the methods of studies on infant ultrasonic production and maternal retrieval in small rodents. *Behav. Genet.* **35:**31–52.

Hofer, M. A., Shair, H. N., Masmela, J., and Brunelli, S. A. (2001). Developmental effects of selective breeding for an infantile trait: the rat pup ultrasonic isolation call. *Dev. Psychobiol.* **30**:231–246.

Knutson, B., Burgdorf, J., and Panksepp, J. (2002). Ultrasonic vocalizations as indices of affective states in rats. *Psychol. Bull.* **128**:961–77.

Lykken, D. T., and Tellegen, A. (1996). Happiness Is a Stochastic Phenomenon. *Psychol. Sci.* **7**:186–189.

Mobbs, D., Greicius, M. D., Abdel-Azim, E., Menon, V., and Reiss, A. L. (2003). Humor modulates the mesolimbic reward centers. *Neuron.* **40**:1041–1048.

Panksepp, J. (1998). *Affective neuroscience: The foundations of human and animal emotions.* London:Oxford University Press.

Panksepp, J., and Burgdorf, J. (1999). Laughing rats? Playful tickling arouses high frequency ultrasonic chirping in young rodents. In S. Hameroff, D. Chalmers, and A. Kazniak (eds.), *Toward a science of consciousness III*, Cambridge, Mass.:MIT Press, pp. 231–244.

Panksepp, J., and Burgdorf, J. (2000). 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. *Behav. Brain Res.* **115**:25–38.

Panksepp, J., and Burgdorf, J. (2003). "Laughing" rats and the evolutionary antecedents of human joy? *Physiol. Behav.* **73**:533–547.

Panksepp, J., Gordon, N., and Burgdorf, J. (2002a). Empathy and the action-perception resonances of basic socio-emotional systems of the brain. *Behav. Brain Sci.* **25**:43.

Panksepp, J., Knutson, B., and Burgdorf, J. (2002b). The role of brain emotional systems in addictions: a neuro-evolutionary perspective and new self-report animal model. *Addiction* **97**:459–69.

Panksepp, J., Moskal, J., Panksepp, J. B., and Kroes, R. (2002c). Comparative approaches in evolutionary psychology:molecular neuroscience meets the mind. *Neuroendocrinol. Lett.* **23** (Suppl. 4): 105–115.

Panksepp, J., Siviy, S., and Normansell, L. A. (1984). The psychobiology of play:theoretical and methodological perspectives. *Neurosci. Biobehav. Rev.* **8**:465–492.

Provine, R. R. (2000). *Laughter:a scientific investigation*. New York:Viking.

Scott, E., and Panksepp, J. (2003). Rough and tumble play in human children. *Aggressive Behav.* **29**:539–551.

Scott, J. P., and Fuller, J. L. (1965). *Genetics and the social behavior of the dog*. Chicago: University of Chicago Press.

Steimer, T., la Fleur, S., and Schulz, P. E. (2001). Neuroendocrine correlates of emotional reactivity and coping in male rats from the Roman high (RHA/Verh)-and low (RLA/Verh)-avoidance lines. *Behav. Genet.* **27**:503–12.

Thornton, L. M., Hahn, M. E., and Schanz, N. (2005). Genetic and developmental influences on infant mouse ultrasonic calling. Patterns of inheritance in the calls of mice 3–9 days of age. *Behav. Genet.* **35:**73–83.

Vanderschuren, L. J., Niesink, R. J., and Van Ree, J. M. (1997). The neurobiology of social play behavior in rats. *Neurosci. Biobehav. Rev.* **21**:309–326.