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Selective Breeding for an Infant Phenotype: Rat Pup Ultrasonic Vocalization (USV)

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To examine processes underlying generational and developmental influences on anxiety, this laboratory produced two lines of (N:NIH strain) rats, selectively bred on the basis of extreme rates of ultrasonic vocalization in 2 minutes of isolation at Postnatal Day 10. The research reviewed in this article focuses on: (1) establishment of the selectively bred lines; (2) defining infant behavioral and physiological phenotypes and (3) determining whether infantile USV phenotypes endure over development. The High and Low lines have diverged widely in their USV rates from each other and from the Random control line, which has maintained N:NIH strain rates overall from generation to generation. Beginning in the 11th generation, High USV pups have shown significantly higher frequencies of defecation and urination during isolation screening than the Low USV and Random control line. Both lines show altered autonomic regulation of heart rates (HR) in response to stressors as juveniles and adults. These differences in HR responses in High and Low lines appear to be mediated by changes in the balance of sympathetic *versus* parasympathetic mechanisms. Other behavioral characteristics of the High line are consistent with an "anxious"/ "depressive" phenotype, such as vocalizations to touch in a novel environment, and performance in the Porsolt Swim, whereas Low line shows few differences in anxiety behavior. Future work will resolve the similarities and differences in the High and Low phenotypes and provide a developmental perspective to the growing body of information about affective regulation in humans and animals provided by selectively bred animal models.

KEY WORDS: Breeding; vocalization; postnatal; development; anxiety; depression; autonomic.

INTRODUCTION

As in much of animal research, the major reason for embarking on a selective breeding program is to provide an animal model for a human disorder. The goal of a selective breeding an animal model is quite specific, which is to model an aspect or a component of a disorder that is believed to depend upon genetic variation in the human population. Animals are bred on the basis of high and low values, respectively, of a relevant trait or phenotype that is expected to show genetic variation in the animal population as well. The expectation is that after many generations, the high and low lines will be "enriched" by genes facilitating the high or low phenotype; in contrast, genes that do not facilitate the selected phenotype will become less frequent over generations, thus increasing differences between lines (Plomin *et al.*, 1991; Snustad *et al.*, 1997). The eventual consequence is that putative genetic linkages between the selected system and other systems are strengthened and revealed in the process (DeFries, 1981). It is at the intersection of these linkages that the real work of the selected lines occurs, which is to understand mechanisms underlying associations between systems in both species (Deitrich, 1993).

Human anxiety and depression disorders display about 10–20% co-morbidity in clinical populations, and show familial inheritance patterns (Warner *et al.*, 1999; Weissman *et al.*, 1996; Wickramaratne *et al.*, 2000). In modeling such

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disorders, the goal of selective breeding lines has been to selectively increase or decrease the incidence of explicit "anxiety-like", or "depressed-like" behavior or physiology (e.g., Overstreet *et al*., 2002). The characteristic chosen in a given selection program is usually based on a standard laboratory-derived measure (e.g., shock, open field) designed to elicit behavior, physiology, or neurochemistry associated with a desired anxiety model. Differences between selected lines in *other* behavioral measures such as immobility or avoidance behavior, plus maze performance, or of central or peripheral autonomic system (ANS) control, hypothalamic-pituitary-adrenal (HPA) axis activation, or changes in plasma or brain neurotransmitters, are taken to mean that one or the other of the lines has been bred for a central state that has been characterized as "anxiety" or "depression".

This selective breeding program was stimulated by human epidemiological/clinical studies showing that children who at 4 months of age cry more in response to novelty (Kagan and Snidman, 1991), are highly likely to become shy and inhibited in childhood (Biederman *et al*., 1993; Hirshfeld *et al*., 1992; Kagan *et al*., 1987, Rosenbaum *et al*., 1993). Inhibited children show stability in behavior as they grow older, and are more likely to manifest anxiety disorders in late childhood, and anxiety and depression disorders in adolescence and adulthood (Hirshfeld *et al*., 1992; Schwartz *et al*., 1999, 2003). Likewise, children of parents diagnosed with panic disorder and depression are at greater risk for childhood separation and anxiety disorders (Biederman *et al*., 2001; Rosenbaum *et al*., 2000; Warner *et al*., 1999) and also to exhibit higher rates of behavioral inhibition (Rosenbaum *et al*., 1991). These studies are consistent with: (1) inherited "biases" or traits conferring risk for anxiety/depressive disorders in affected individuals; and (2) stability in the course of such biases that can be traced through development.

Developmental studies suggest that physiological correlates of anxiety are stable, beginning early in life. During a maternal stressor, fetuses of highly anxious women show significant heart rate (HR) increases consistent with autonomic nervous system (ANS) dysregulation, whereas no changes in HRs are observed in fetuses of non-anxious women (Monk *et al*., 2000). Children who remain shy and inhibited show higher HRs and lower HR variability, suggesting that the association between emotional/behavioral regulation and ANS dysregulation is maintained from early to mid-childhood (Biederman *et al*., 1990; Kagan, 1987; Kagan *et al*., 1989; Kagan and Snidman, 1999). Children diagnosed with anxiety disorders are significantly more likely than controls to show decreased HR variability underscoring putative links between anxiety disorders and ANS dysregulation (Yeragani *et al*., 2001).

The developmental research model historically used in our laboratory has been the separation of infant rats from mother and littermates, which has provided profound insights into the biological and behavioral regulation of infant development by the mother, and cardiovascular regulation in particular (Hofer 1984, 1994). In the past few years this research has focused on the vocalization response to separation in infant rats as a model of affective regulation by the mother (Hofer, 1996). In order to create such a developmental-genetic model system, we used a novel modification of the selective breeding strategy in which the selected trait was an infantile rather than an adult behavior. The idea was to manipulate age-specific gene expression to test hypotheses that behavioral temperamental differences and autonomic dysregulation in infancy might predispose animals to exhibit behavioral and/or autonomic nervous system dysregulation in adulthood (Brunelli and Hofer, 2001; Brunelli *et al*., 2002).

INFANT BEHAVIORAL RESPONSE TO SEPARATION IN RATS: RAT PUP ULTRASONIC VOCALIZATION

When displaced from the home nest and separated from mother and littermates, the infant rat (pup) immediately begins to vocalize, a behavior called "protest", characteristic of most mammalian infants (Bowlby, 1969). The vocal response of isolated rat pups consists of high-frequency (40–50 kHz) ultrasonic vocalizations (USV) that elicit maternal search and retrieval (Brunelli *et al*., 1994; Noirot, 1972; see also, Brudzynski; Hahn and Lavooy, in press). Rat pups, like other mammalian infants, experience maternal separation as a stressful event, as measured by ANS and cardiovascular changes, HPA axis indices, and noradrenergic activity (Hennessy and Weinberg, 1990; Hofer and Reiser, 1969; Harvey and Hennessey, 1995). The co-occurrence of these systems with USV suggests that USV is a behavioral component of a coordinated defense system in rat pups. As an animal model rat pup USV has provided insights into the

development and neurobiology of anxiety (Hofer, 1996; Insel and Winslow, 1991).

Neuroanatomical studies have linked USV in both rat pups and adults to research on fear and anxiety. The expression of infant USV is controlled by the same central system sites as adults, e.g., the periaqueductal gray (PAG, Goodwin and Barr, 1997; Graef, 1994). For the most part, rat pup USV is mediated by the same neurotransmitter systems as adult USV, suggesting developmental continuity in neurochemical mechanisms. Drugs having anxiolytic actions at γ-aminobutyric acid (GABA), opioid, and serotonin (5HT1A) receptors potently decrease rates of infant and adult USV in aversive situations. Conversely, antagonists for these systems increase rates of USV (Carden and Hofer, 1990; Carden *et al*., 1991; Joyce and Carden, 1999; Olivier *et al*., 1998a, b). Selective serotonin reuptake inhibitors (SSRIs) reduce USV in rat pups (e.g., Joyce and Carden, 1999; Olivier *et al*., 1998b) in parallel with their effects on USV in adult rats, suggesting similar mechanisms of action. Compounds that oppose actions of excitatory amino acids increase USV rates (e.g., NMDA: Kehne *et al*., 1991; Winslow *et al*., 1990).

Despite its utility, what was missing from this experimental paradigm was a model for genetic and infantile risk factors for anxiety-like behavior in adults that would model continuity of disorders in human populations. The possibility that USV rates might be an early expression of a heritable trait related to anxiety in rats was first raised by Insel and Hill (1987), because infant USV rates of the Maudsley-reactive (MR) strain of rats were significantly higher in the first postnatal week than those of the Maudsley-non-reactive (MNR) strain. The result was consistent with adult characteristics of the MR and MNR strains, selectively bred for adult expression of ''emotionality'' and ''non-emotionality'', measured as extremes of defecation and middle-square crosses in open-field tests (Blizard, 1981). Given these results, and since rat pups from different litters vary considerably in rates of calling, and even within litters pups show individual differences (Graham and Letz, 1979), it seemed likely that pup USV was malleable to selective breeding for differential rates of calling (Brunelli *et al*., 1997).

Behavior-genetic studies have examined gene influences on infant USVs in inbred mice, using statistical modeling and cross-breeding techniques. Whitney *et al*. (1978), studying cold-induced USVs in one strain of mice, reported a very small number of genes showing directional dominance affected USV rates. In subsequent studies examining panels of inbred strains, Hahn *et al*. (1987, 1997), Roubertoux *et al*. (1996) and Thornton *et al*. (2005) have provided evidence for additive effects as well, and for epistatic interactions in polygenic systems.

SELECTIVE BREEDING FOR ISOLATION-INDUCED RAT PUP ULTRASONIC VOCALIZATION

Accordingly, our laboratory undertook selective breeding for two lines of rats showing extreme rates of USV (High and Low USV lines) in response to maternal separation and isolation in infancy (Brunelli *et al*., 1997). The research reported in this article has focused on: (1) establishment of the selectively bred lines; (2) defining infant behavioral and physiological phenotypes (3) determining whether infantile USV phenotypes endure over development.

Design of the Selective Breeding Program

In selective breeding for ultrasonic vocalizations the National Institutes of Health (N:NIH) strain rats were used as the foundation strain rather than the Wistar strain used for all of our previous work. The N:NIH strain was developed from eight inbred laboratory strains to represent a broad range of the Rattus norvegicus genotype, ensuring a large and normally distributed basis of alleles to respond to selection pressure, and therefore to yield the largest possible number of phenotypic responses (Hansen and Spuhler, 1984). From the original 25 breeding pairs furnished by the NIH, three laboratory-born progenitor generations (PR1,2,3) were randomly bred in order to characterize USV rates for the N:NIH strain before selective breeding, and to examine the effects of factors known to influence USV at different postnatal ages (Brunelli and Hofer, 1996).

Preliminary Studies: Progenitor (PR) Generations

Prior to selective breeding, USV was measured in the first progenitor generation (PR1) of laboratory-born N:NIH pups. Pups were tested in 2 minutes of isolation over development at postnatal (P) 3, 10, 15 and 18 days. Average rates of USV during isolation at room temperature were highest at 3–4 days, went to half of that rate at 10 days, declining

a further quarter of that rate at 15 days, to nearly zero at 18 days, a trend found in about half of published longitudinal studies (Brunelli *et al*., 1996).

At P3 days naturally occurring ambient temperature variations significantly affected USV rates. Otherwise, temperature variation was not correlated with USV rates in older pups, consistent with earlier developmental studies. The effects of litter as a factor on USV steadily declined over the postnatal period, suggesting that as they become older, USV rates of individual pups are less susceptible to maternal and other influences that act upon the entire litter. Variance in USV accounted for by litter factors in the PR1 generation ranged from about 75% at 3 days of age to about 15% at 18 days of age, with about 36% at 10 days of age. USV rates at 10 and 15 days of age were significantly correlated, suggesting a developmental plateau in the control of USV between these ages; rates from 3 to 10 days were positively, but not significantly correlated (Brunelli *et al*., 1996). Overall, the findings from this study suggested that development of USV over the postnatal period in the N:NIH strain was reasonably consistent with others tested, and pointed to the existence of individual differences traceable to the second postnatal week.

In a separate study USV was measured only at P 10 days of age in all three progenitor generations (PR1, PR2, PR3), consisting of 532 pups in 81 litters (Brunelli and Hofer, 1996). A heritability estimate, based on regression of litter mean USV rates on dam rates for the PR2 and PR3 generations was 0.25, again predicting the susceptibility of USV to significant change under selective pressure. One purpose of the study was to determine baseline vocalization rates in the foundation generation to compare selected rates. A second purpose was to detect associations among variables measured in isolation that might suggest correlated phenotypes with USV at that particular age. Evaluation by principal components factor analysis revealed four factors that corresponded roughly to patterns indicative of: (1) thermoregulatory responses, characterized by high positive loadings of ambient and body temperature variables without USV; (2) a "maturity" factor composed of pup weight, rearing, and urinate/defecate in which older, heavier pups reared and defecated more; and (3) an "anxiety" or "emotionality" factor consisting of USV, D/U (defecate/urinate) and self-grooming; and (4) a mixed locomotor-maturity factor composed of square crosses, temperature and weight.

Mechanics of Selective Breeding

The phenotype selected was based on the number of vocalizations (USVs) emitted by 10-day-old pups in 2 minutes of isolation at room temperature. Pups of the PR3 generation founded the Random, High and Low USV lines. At weaning, 6 offspring (3 males, 3 females) from litters born to PR2 dams (descended from each of the 25 original NIH breeders) were chosen as line breeders, as described below.

Within litters, the first two Random control breeders (one male, one female) were picked using a random number table, then littermate males and females having the highest and lowest rates of USV at PN 10, respectively, were chosen to initiate the High and Low lines. At 80–100 days, females were bred with similarly chosen males from other litters: Random–Random; High–High, and Low–Low. Succeeding selected generations (S1, S2,…,S_n.) are offspring of these initial High, Random and Low USV line breeders. The lines are maintained separately as closed breeding systems, and mating occurs only within lines. Each closed line is made up of 18–19 families, derived from each of the original 25 breeding pairs. Breeding across families within the lines is practiced in order to minimize inbreeding, and also to maximize the number of genes influencing USV within each line (Crabbe *et al*., 1990; DeFries, 1981). The randomly bred line performs the added functions of monitoring for the effects of genetic drift and of environmental changes across generations (Crabbe *et al*., 1990; DeFries, 1981). In practice, this means that breeders are mated with partners from litters outside the natal families, and partners do not share parents or grandparents in common. Outbreeding has also acted to minimize maternal effects (Brunelli *et al*., 2001).

The High and Low lines have diverged widely in their USV rates from each other and from the Random line, which has maintained N:NIH strain rates overall from generation to generation (Fig. 1). Realized heritability values for the High and Low lines over 21 generations of selection were 0.24 and 0.06, respectively.

In a review of this work, Roubertoux (2001) observed that the rapid response to selection in the High and Low lines implied large genotypic and phenotypic variances in the original N:NIH strain population. This, in turn suggested either a lack of adaptive value for USV rate, or stabilizing selection

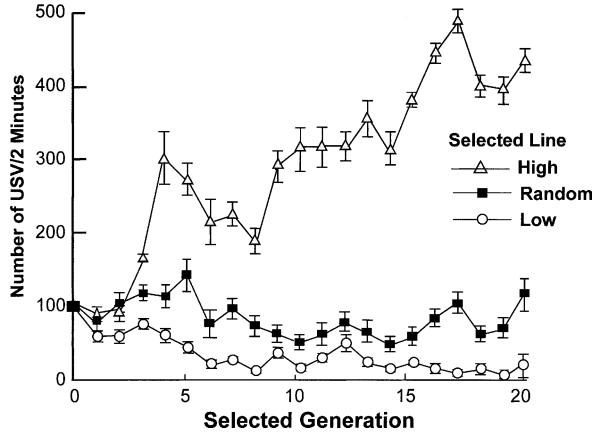

Fig. 1. Shows the results of selective breeding across 20 generations in High, Low and Random (control) lines. The high or low phenotype selected was based on the total number of USVs emitted during 2-minutes isolation at room temperature. The High line is indicated by filled triangles, the Low line by open circles, and the Random line by filled squares. Values are for litter means:the number of litters tested per generation in each line ranged from:14–39 (median = 28) for Highs; 17–32 (median = 22) Randoms; 14–44 (median = 28.5) Lows.

against extremes of rates favoring heterozygotes, in concurrence with mouse data. In addition, the gradual nature of response to selection over generations in the lines was thought to be consistent with mouse data (see Hahn and LaVooy review, in press) suggesting that USV is a product of small gene effects with numerous links to other genes also having small effects (Roubertoux, 2001). Alternatively, the rapidity of selection effects in the first few generations suggests a few alleles having large effects (Henderson, personal communication).

Based on the foregoing evidence, including the earlier PR generation studies, we hypothesized associations of USV with other systems mediating anxiety and with autonomic nervous system regulation.

ASSOCIATIONS AMONG BEHAVIORS IN ISOLATED PUPS ACROSS GENERATIONS

As noted in the Progenitor generations (PR1–PR3), principal components analysis of data generated by isolated pups had revealed that USV and D/U loaded positively, and self-grooming loaded negatively on the third principal component (Brunelli and Hofer, 1996). A strong and stable association between USV and D/U (but not self-grooming) was maintained in the Random and High lines in the 9^{th}–12^{th} generations (Brunelli and Hofer, 2001).

Table I shows phenotypic correlations based on standardized scores for the same variables in recent generations screened at P10 (generations 15–21). In this combined sample and in the smaller Random line sample, USV and D/U were modestly but significantly correlated. This was not true for the High and Low lines, presumably because their distributions of vocalization rates had become more truncated with selection. Moderate, significant correlations were seen within all groups between pup body weight and post-test axillary temperature, probably reflecting better thermoregulatory capabilities in larger animals. Note also that across groups (ALL) all variables seen in isolation (USV, D/U, self-grooming, squares crossed and rearing) showed low but significant correlations with weight, and in some instances with temperature (self-grooming, rearing). The correlations of these variables with weight were indeed reflected in a significant multivariate covariate effect of weight, $F(1,545) = 121.250, p < 0.001$.

Table II shows the results of principal components analyses based on the phenotypic correlations, in the combined sample across the three lines, and separately in the Random, High and Low lines. In the combined sample (IIA) and in the Random line (IIB) USV and D/U appear with high loadings on the second component, orthogonal (uncorrelated) to the first. The component characterized by USV and D/U replicates the same component found in the PR generations prior to selection (Brunelli and Hofer, 1996), and in Random line pups in the combined S9–S12 generations, but not in the High line (Brunelli and Hofer, 2001). The reliability of this association in the Random line across generations of closed breeding (with the inevitable loss of heterogeneity through drift over time; DeFries, 1981), is evidence that enough genetic variation has been maintained in this population to allow the detection of this association.

The correlations among body weight, body temperature and rearing shown in Table I in all three lines appear as moderate to high loadings on principal component 1 both across lines (II, A) and within lines (II, B, C, D). The commonality underlying these functions is maturity, in that larger animals maintain higher body temperatures and do more rearing, as found in previous generations as well. However, self-grooming and square crossing variables appear in various combinations either on the same component as body weight and axillary temperature or on a separate (orthogonal) component. Presumably, these changing associations reflect the relative amounts of shared *versus* non-shared variance among these variables, and

Table I. Phenotypic Correlations Among Variables[a] Measured in Isolation In Generations 15 to 21

Variable in 2-min isolation	Selected line	[b]D/U	Self-groom	[c]Square cross	Rear	Weight	[d] Axillary temp‡.
USV	ALL	**0.276*****	−0.001	−0.018	−0.032	0.160**	0.115
	Random	**0.273****	−0.091	−0.024	−0.150	−0.060	−0.090
	High	0.060	−0.150	−0.016	−0.182	−0.048	−0.086
	Low	0.016	−0.034	−0.033	−0.056	−0.121	−0.154
D/U	ALL		**0.222*****	−0.022	**0.161****	**0.150****	0.095
	Random		0.222	0.153	0.187	0.133	0.091
	High		**0.317*****	0.021	**0.142***	0.106	0.025
	Low		0.085	−0.038	0.211	0.152	0.095
Self-groom	ALL			−0.021	**0.153****	0.136*	**0.154****
	Random			0.044	0.167	0.074	0.049
	High			0.020	**0.216***	0.174	0.143
	Low			−0.091	0.104	0.187	0.202
Square cross	ALL				**0.184*****	**0.149****	0.081
	Random				**0.356*****	0.172	0.088
	High				0.055	0.146	0.126
	Low				0.185	0.178	0.047
Rear	ALL					**0.339*****	**0.163****
	Random					**0.337*****	0.106
	High					**0.293*****	0.141
	Low					**0.415*****	**0.209***
Weight	ALL						**0.381*****
	Random						**0.413*****
	High						**0.334*****
	Low						**0.380*****

Based on litter means: ALL $N = 549$; Random $N = 144$; High $N = 194$; Low $N = 211$.
[a]Based on average z-scores; [b]Defecation/urination; [c]Number of squares crossed; [d]Axillary temperature in °C.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

suggest the fluidity of their functions in isolated pups.

LINE DIFFERENCES IN AUTONOMIC REGULATION

Based on associations found between USV and D/U in the founding population and in selected generations, we hypothesized that D/U would increase with USV rates in High line pups over generations. When tested in the fifth selected generation (S5), analyses of litter means revealed no line differences in behaviors or physiological variables measured other than USV at that point (Brunelli *et al*., 1997). Beginning in the 11th generation, however, the High line has shown significantly more D/U in isolation, $F(2,133) = 6.980$, $p < 0.001$, Fig. 2a. This difference has been maintained ever since, Line F (2,1048) = 21.028, $p < 0.001$; Generation (S11–S20) X Line $F(36,1048) = 1.820$, $p < 0.01$.

The associations found between USV and D/U suggest underlying genetic correlations between the two phenotypes but could be due to nongenetic factors such as the co-occurrence of functional systems related to anxiety. Fig. 3 shows the comparison of z-score units for D/U and USV in recent generations (15–21) of High and Low line pups. Whereas the mean difference in D/U between the lines is less than 1 SD unit (0.625), in comparison, the mean difference between the lines for USV is 2.54 SD units, suggesting that these two traits are genetically uncorrelated.

Defecation has long been considered a measure of emotionality in rodents (Gray, 1972, 1979) although this interpretation has been challenged for just as long (Archer, 1973). Factor analytic studies of rodents in different "anxiety" tests have consistently shown only a loose association of defecation with other anxiety measures (Courvoisier *et al*., 1996; Ramos *et al*., 1997; Ramos *et al*., 1998). In common with these statistical analyses, recent QTL analyses in a large sample of F2 intercrosses between the DeFries strain of mice selected for differences in open-field activity suggested that

Table II. Principal Components Analyses on Variables Measured in Isolation: Generations 15 through 21: Based on Litter Means

	Principal components[a]	
Variables	1	2
A: ALL lines (N = 549 litters)		
USV	−0.090	**0.726**
D/U	0.116	**0.730**
Square cross	**0.536**	−0.271
Self-groom	0.263	**0.381**
Rear	**0.708**	0.017
Body weight	**0.704**	0.271
Axillary temperature	**0.556**	0.269
% Variance	24.057	20.355
Eigenvalue	1.684	1.425
B: Random line (N = 144 litters)		
USV	**−0.300**	**0.734**
D/U	0.271	**0.799**
Square cross	**0.536**	−0.271
Self-groom	**0.310**	0.265
Rear	**0.706**	0.092
Body weight	**0.728**	−0.037
Axillary temperature	**0.571**	−0.145
% Variance	26.962	18.786
Eigenvalue	1.887	1.315
C: High line (N = 194 litters)		
USV	**−0.318**	−0.098
D/U	−0.101	**0.784**
Square cross	**0.484**	−0.173
Self-groom	0.168	**0.753**
Rear	**0.466**	**0.423**
Body weight	**0.706**	0.192
Axillary temperature	**0.702**	0.008
% Variance	22.609	20.542
Eigenvalue	1.583	1.438
D: Low line (N = 211 litters)		
USV	−0.026	−0.037
D/U	0.269	**0.368**
Square cross	**0.494**	**−0.671**
Self-groom	0.191	**0.694**
Rear	**0.718**	0.002
Body weight	**0.780**	0.144
Axillary temperature	**0.569**	0.354
% Variance	26.729	17.365
Eigenvalue	1.871	1.216

[a]Principal components analyses were used for initial extraction followed by varimax rotation. The number of eigenvalues accepted were specified by two criteria: (1) eigenvalue > 1.0; and 2) the "scree" test (Cattell, 1965), in which factoring stops at the point at which the eigenvalues "slope" begins to level off. Loadings on components => 0.30 (Henderson *et al.*, 2004) are noted in bold.

a genetic correlation between autonomic reactivity and anxiety measures was largely absent (Henderson *et al.*, 2004). Similar analysis in the Roman High and Low Avoidance lines of rats detected

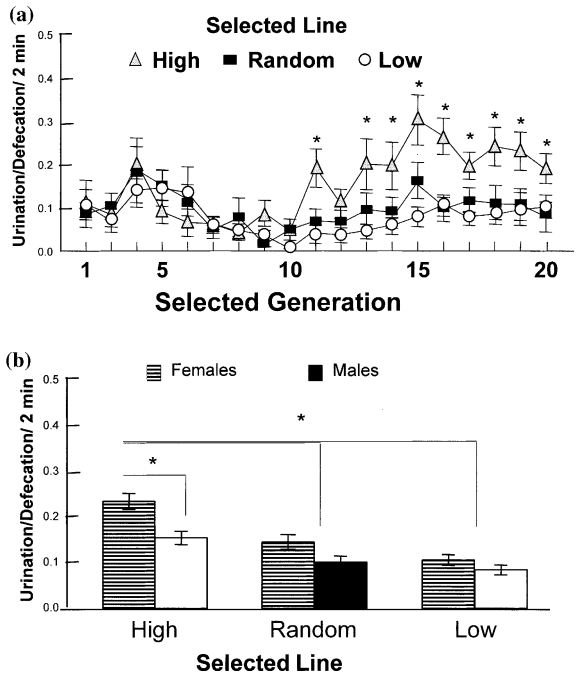

Fig. 2. (a) Frequency of defecation/urination over 20 generations in High and Low USV and Random line pups. High line pups defecated and urinated significantly more, beginning in the 11th generation. (b) Male-female differences in defecation/urination collapsed over 20 generations in High and Low USV and Random line pups. Overall, all female pups defecated more than males; High line female pups defecated and urinated significantly more than males.

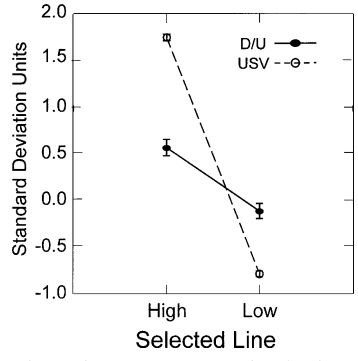

Fig. 3. Comparisons between USV and D/U in High and Low USV lines in standard deviation units in generations 15–21.

one QTL specific to defecation that bore no relationship to other measures of fear derived from a conditioned fear session (Fernandez-Teruel *et al.*, 2002). However, Antoniadis and McDonald (1999), reported that whereas only one session was required to condition freezing to shock, large individual differences in defecation, ultrasonic vocalization and HR responses required several sessions to

condition, suggesting that some behavioral and physiological responses must be recruited over a longer period of time in order to be expressed. Such a model would explain the dissociation of defecation/urination from other, more immediate measures of anxiety, and fits the etiology of human stress-related physical disorders that sometimes take years of sensitization to emerge (Sloan *et al.*, 1999).

D/U was the only gender-linked difference found in the postnatal period: overall, female pups eliminated more than males in isolation, $F(1,1462) = 13.295$, $p < 0.001$, although only females in the High line showed significantly more D/U than males in their respective line, Sex X Line $F(2,1462) = 3.280$, $p < 0.05$ (Fig. 2b). High line female pups eliminate more in isolation than both Random and Low line female pups as well, suggesting that higher levels of D/U may be sex-linked. Recently, QTLs on X-loci have been found to influence anxiety behavior patterns in mice and rats (Ahmadiyeh *et al.*, 2003; Henderson *et al.*, 2004; Yoshikawa *et al.*, 2002). Specifically QTLs for D/U have been located on the X-chromosome with reasonably high LOD scores ranging from 3.7 to 11.7 (Henderson *et al.*, 2004), suggesting a possible locus for gender-related differences.

Although highly statistically significant, it is not clear whether small differences in D/U in infant rats have biological significance. Nor is it clear whether differences so early in life are linked in any way to central or peripheral structures and systems governing D/U later in life. D/U is complexly regulated by both the sympathetic and parasympathetic systems (Blok and Holstege, 1999), with links to central structures and neurotransmitter systems responsive to stress (e.g., corticotropin-releasing hormone (CRH) projections; Nuding *et al.*, 1998; Valentino *et al.*, 1994). Early adverse experience alters later visceromotor functioning in rats along with behavioral and endocrinological systems, probably through changes in CRF release in infancy (Coutinho *et al.*, 2002). Thus, animals predisposed to greater gastric motility in infancy are likely to be good candidates for greater susceptibility to the effects of stress in the modulation of autonomic systems later in life. The sex-biased nature of gastro functioning in the lines may bear some functional relationship to sex-biased dysregulation of gastro-intestinal function in animal models (Ramos *et al.*, 1999; Stam *et al.*, 1997, 1999) and in women suffering from anxiety disorders (Heaton *et al.*, 1992; Lydiard *et al.*, 1994).

CARDIAC AUTONOMIC REACTIVITY DIFFERENCES

Since D/U is complexly regulated by both the sympathetic and parasympathetic systems, this finding prompted examination of ANS regulation of HR in the selected lines. Increased HR reactivity to environmental challenge is associated with behavioral reactivity in animals, and with anxiety syndromes in humans, so we therefore predicted exaggerated HR changes to isolation in High line (Brunelli *et al.*, 2002). At P 10 the ANS is not fully functional, and there is little or no parasympathetic activity to modulate sympathetic influences on HR to stress (Hofer and Reiser, 1969). However, by late in the third postnatal week when HR is regulated at nearly adult levels, we found that at P 18 High line HRs were significantly higher than Random line as predicted. What was surprising was that Low line HRs were significantly higher than both Random and High lines (Fig. 4). Moreover, compared to Random line pups, when returned to the home cage, High and Low line HRs did not fall to baseline levels, indicating that cardiac reactivity was maintained long past the stressor. Pharmacological blockade of sympathetic and parasympathetic systems revealed

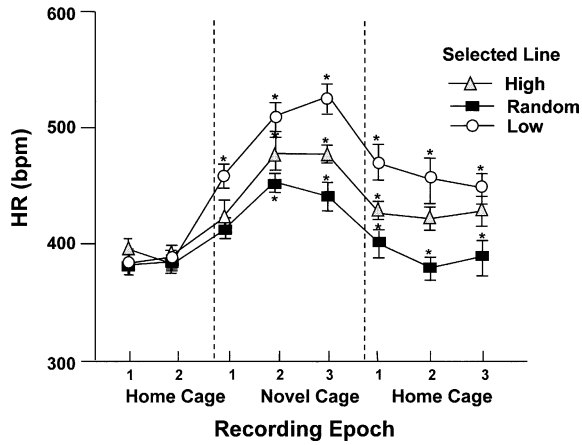

Fig. 4. Average heart rates (HRs, in beats per minute [bpm] of Postnatal Day (P) 18 juveniles in the High, Low, and Random USV lines. Test Epochs represent 2-min intervals from home cage baseline (Base) in the home cage (between first and second vertical, dotted lines), to isolation (Is 1 - Is 3: right of first vertical, dotted line) in a novel cage, to recovery (Rec 1 - Rec3) back in the home cage (right of second vertical, dotted line). Reprinted by permission from the American Psychological Association, Inc.

that HR reactivity in P18 High and Low lines was subserved by different ANS control. High line pups' elevated HRs were the result of greater sympathetic reactivity, whereas Low pups' even higher HRs were due to profound parasympathetic withdrawal, coupled with greater than normal sympathetic reactivity. This finding established for the first time that in response to selection pressure, *both lines* showed alterations in another system theoretically related to affect regulation, and that the Low line was equally, if not more reactive to stress.

In adult testing to 30 minutes of restraint stress, HR differences have demonstrated continuity with juvenile HR responses. Compared to the Random line High and Low line males demonstrate higher HRs, which are maintained over the entire restraint period (Fig. 5). Thus, selection appears to have predisposed individuals to exaggerated HR reactivity later in life as well. Profiles of higher HRs differentially modulated by the two branches of the ANS can be seen in two distinctly different human clinical populations as well:those with anxiety disorders exhibiting high HRs corresponding to greater sympathetic reactivity (Yeragani, 1995), as opposed to those clinical groups characterized by hostility and aggression that show higher HRs mediated by lower parasympathetic antagonism (Pine *et al.*, 1998; Sloan *et al.*, 1994, 1999). These human disorders suggest a hypothetical behavioral correlate of parasympathetic regulation that can be tested in adult social and aggressive interactions in the lines.

Control of cardiovascular reactivity during stress is the result of reciprocal interactions of the ANS directed by central nervous system processes (Berntson *et al.*, 1994). However, we do not yet know whether central processes are involved in the regulation of ANS control of cardiovascular system responses to stress in the selected lines. Clearly, the balance of interactions within the ANS has been altered in both High and Low USV lines. Hypothetically, if central processes were involved in "top down" regulation of autonomic and cardiovascular responses, then animals should show greater behavioral responses consistent with anxiety or reactivity to laboratory stressors.

Preliminary data suggest that some standard laboratory tests reveal differences between lines, while others do not. In general, the High line demonstrates more behavioral indices consistent with an "anxious"/"depressed" phenotype, whereas Low line has shown few behavioral differences from Random controls. As shown in Fig. 6a, adult High line males emit more USVs in response to tactile stimulation in a novel cage (Brudzynski, in press; Brudzynski and Ociepa, 1992) and also more time immobile in the Porsolt test (Fig. 6b), indicating

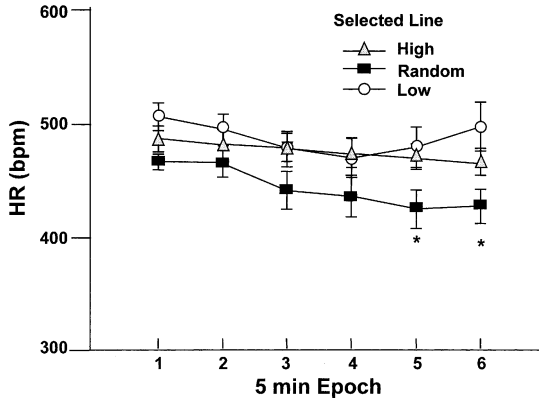

Fig. 5. Adult male mean HRs (± SEM) over 30-min of restraint at 10-min intervals.

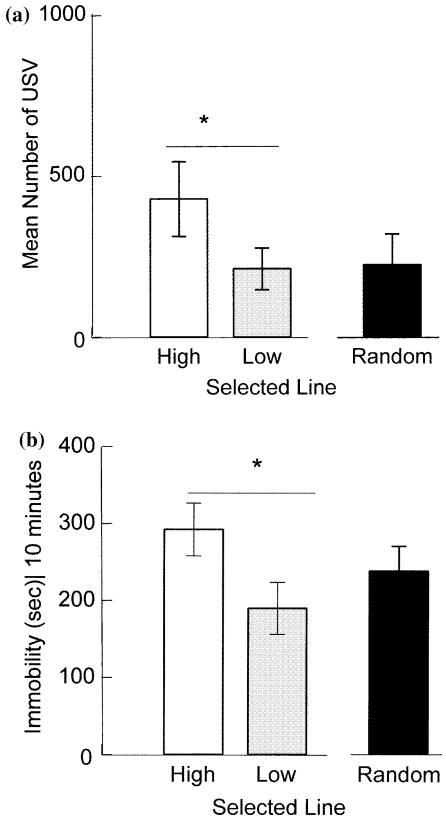

Fig. 6. Responses of adult males from the High, Low and Random lines to two different stressors: (a) Number of vocalizations during a 5-minute period in a novel test chamber in response to human touch on the back of the neck. (b) Amount of time spent immobile during a 10-minute period in the Porsolt Swim.

that the High line is susceptible to certain types of stressors (Shair *et al*., 2000).

On the other hand, the High line has shown erratic performances in the elevated plus maze over the generations, inconsistent with an ''anxiety'' phenotype (Dichter *et al*., 1994; Rojowski *et al*., 1999; Shair *et al*., 2000). Our results correspond to a growing number of negative plus maze results shown by lines of selected rats, for example the Maudsley Reactive strain (Blizard and Adams, 2002) and strains selected for cholinergic or serotonin responsiveness (Overstreet *et al*., 2003). Inconsistency in behavioral tests, however, are to be expected if different behavioral tests encapsulate only one or more dimensions of a multidimensional construct like anxiety. Factor analyses of elements of behavior and physiology in animal tests overwhelmingly yield multiple, independent factors (e.g., orthogonal principal components for locomotion, open arm entries, defecation), suggesting that different measures probably reflect different underlying biological mechanisms (Maier *et al*., 1988; Ramos *et al*., 1997). However, a recent QTL analysis identified five genetically separable dimensions of anxiety that cut across behavioral tests, and were influenced to a greater or lesser extent by loci at number of chromosomes. One of these dimensions was autonomic reactivity (Henderson *et al*., 2004). Such approaches hold the promise of elucidating mechanisms underlying individual variation in systems associated with emotional regulation.

Another, increasingly common way of approaching the problem of correlated phenotypes is to compare anxiety phenotypes across selectively bred strains bred for different facets of a trait. As noted by Henderson (1997), between-lab replications of selected traits provide evidence of external validity across lines, laboratories, environmental variations and measures. As the number of selected rat strains modeling specific psychobiological aspects of emotional regulation, such as anxiety and depression have proliferated, this strategy has become possible in anxiety research (see Blizard and Adams, 2002; Overstreet, 2002; Ramos and Mormede, 1998 for reviews). Indeed, highly inbred strains originally bred for other characteristics like the Spontaneously Hypertensive Rats and their control strain, the Wistar-Kyoto (high and low blood pressures), share many of the same behavioral characteristics as high-low anxiety/depression lines (e.g., Durand *et al*., 1999). Numerous sublines based on anxiety-like characteristics shown by different strains have been generated to test hypotheses about common relationships among anxiety variables (e.g., Fernandez-Teruel *et al*., 2002; Ramos *et al*., 1997, 2003).

Ramos and Mormede (1998) point out that each strain is a result of its distinct genetic background and of the methods of selection and breeding used, and thus presents a unique set of neurobiological and behavioral parameters. Hence, selection for a given phenotype may independently co-select other contributing factors that are not linked genetically but which contribute to a phenotype, for example, salt sensitivity and hypertension in the SHR strain (Mormede *et al*., 2002; Myers 1992). The end result may be that selectively bred models presenting such unique combinations may provide better real-world models of clinical syndromes, in which such functional links are concentrated in small subpopulations of individuals. The DSM-IV categorizes numerous clinical groups that display specific subtypes of anxiety disorders (e.g., female patients with anxiety disorders also manifesting intestinal disorders) Lydiard *et al*., 1994; Stam *et al*., 1999.

With the proliferation of lines selected for aspects of anxiety-like traits, some striking similarities have emerged across lines. Broadly stated, in a given breeding program one or the other of the selected lines exhibits a ''passive'' coping style, and the other an ''active'' coping style (De Boer *et al*., 2003; Koolhaas *et al*., 1999; Million et al., 2000; Overstreet 2002; Ramos and Mormede 1998). In general, the passive style is manifested in the line bred for the more ''emotional'', ''depressed'' or ''anxious'' criteria, and may include more freezing, lower activity, defecation, immobility in the Porsolt swim. On the other hand, the ''active'' line bred for the other extreme of the targeted response, often exhibits greater reactivity in response to threat or stress, which frequently (but not invariably) includes more flight behavior, aggressiveness, behavioral activity, and greater reactivity in autonomic, cardiac and catecholamine systems. Each of these coping styles carries its own psychological and physiological strengths and risks in the short- and in the long-term (de Boer *et al*., 2003; Koolhaas *et al*., 1999; Sloan *et al*., 1999). Systematic studies are needed to determine the commonalities and differences across lines in order to begin to characterize the genetic architecture that supports them.

Based on data so far, the High line shows reasonable stability in behaviors and systems associated with anxiety: higher USV rates in infancy and adulthood, and increased HR reactivity to stress as

juveniles and as adults. High line males also show a more "depressed" profile in the Porsolt Swim test, although not more anxious-type behavior in the plus maze, a standard anxiety test. Thus, the High line may prove to be the first developmental model for a "passive" phenotype at risk for adverse outcomes from infancy into adulthood. In contrast, Low line adults show little or no anxiety by most laboratory criteria. Yet Low line juveniles and adults show higher HRs to stress, mediated by extreme parasympathetic withdrawal. Though seemingly paradoxical, autonomic regulation in the Lows is consistent with an "active" autonomic profile and could be predicted to be more aggressive in social interactions as well as to show cardiovascular risk (Everson *et al*., 1998, 1999; Koolhaas *et al*., 1999). Based on such findings, specific hypotheses can now be tested regarding behavioral, autonomic and cardiovascular systems in the two lines. Bringing a unique developmental perspective to selective breeding, the High/Low USV lines will add to the growing body of research on mechanisms underlying inherited affective regulation in humans and animals provided by selectively bred lines.

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