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Selective Breeding for Infant Rat Separation-Induced Ultrasonic Vocalizations: Developmental Precursors of Passive and Active Coping Styles

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Abstract

Human depression and anxiety disorders show inherited biases across generations, as do antisocial disorders characterized by aggression. Each condition is preceded in children by behavioral inhibition or aggressive behavior, respectively, and both are characterized by separation anxiety disorders. In affected families, adults and children exhibit different forms of altered autonomic nervous system regulation and hypothalamic-pituitary-adrenal activity in response to stress. Because it is difficult to determine mechanisms accounting for these associations, animal studies are useful for studying the fundamental relationships between biological and behavioral traits. Pharmacologic and behavioral studies suggest that infant rat ultrasonic vocalizations (USV) are a measure of an early anxiety-like state related to separation anxiety. However, it was not known whether or not early ultrasound emissions in infant rats are markers for genetic risk for anxiety states later in life. To address these questions, we selectively bred two lines of rats based on high and low rates of USV to isolation at postnatal (P) 10 days of age. To our knowledge, ours is the only laboratory that has ever selectively bred on the basis of an infantile trait related to anxiety. The High and Low USV lines show two distinct sets of patterns of behavior, physiology and neurochemistry from infancy through adulthood. As adults High line rats demonstrate “anxious”/“depressed” phenotypes in behavior and autonomic nervous system (ANS) regulation to standard laboratory tests. In Lows, on the other hand, behavior and autonomic regulation are consistent with an “aggressive” phenotype. The High and Low USV lines are the first genetic animal models implicating long-term associations of contrasting “coping styles” with early attachment responses. They thus present a potentially powerful model for examining gene-environment interactions in the development of life-long affective regulation.

Temperament and attachment in children: developmental stability and inheritance

Behavioral inhibition in childhood is a pattern of responding in which the child shows anxiety, distress or caution in response to novelty [16;17;78;79]. Behavioral inhibition has been suggested to be a *temperamental trait*, defined as biologically-based stability of affect and behavior over the course of childhood through adolescence and into adulthood [133]. Thus, toddlers exhibiting behavioral inhibition continue to show behavioral inhibition as children, and are far more likely to manifest anxiety disorders as adolescents, perhaps as a consequence of or as a continuation of this temperamental trait [16,79,133]. Adults that had been categorized as inhibited in childhood exhibit greater functional MRI response in the amygdala to novel

versus familiar faces, consistent with greater fear to novelty and anxiety, and suggesting that behavioral inhibition represents a lifelong trait with neurophysiological correlates [134].

In parallel with temperamental predisposition, insecure attachment behavior is also associated with inhibition and anxiety. Infants who at 4 months were categorized as insecurely attached, Anxious/Ambivalent are more likely to be inhibited and anxious in childhood [13,83,84,138,152]. Infant and child attachment classifications are associated with specific parenting styles, signifying that even the earliest manifestations of individual differences in behavior must also be considered in the context of parent-child interactions [96,148]. Regardless of origin, pathologically increased separation anxiety in children has been associated with earlier onset of adult anxiety/depressive disorders [13,128,134,152,154,155].

A significant percentage of children of mothers diagnosed with anxiety disorders exhibit insecure, Anxious/Ambivalent attachment, behavioral inhibition, or anxiety disorders [41,95]. Likewise children of parents with panic or major depressive disorder exhibit higher rates of behavioral inhibition [16,17] and are at greater risk for childhood anxiety and depressive disorders [166-169]. Separation anxiety is also seen more frequently in children from families with cross-generational depressive and anxiety disorders [152,167]. While indicating that early separation anxiety is a marker for early affective dysregulation associated with temperament, these studies also suggest that there exists a relationship between inherited characteristics of the child, and parenting style in families either predisposed to or actively suffering from anxiety and depressive disorders (“gene-environment correlations”) [35,69,130,148]. Moreover, human gene-environment correlations invariably include a wider environment consisting of psychosocial family characteristics, and socio-demographic variables [37,130].

Behaviorally inhibited children show significant differences in heart rates in a variety of situations and across ages [97,151]. Fetuses of highly anxious women show significant heart rate increases during a maternal stressor, whereas the fetuses of non-anxious women exhibit minor heart rate decreases [102-104]. Infant risk for depression and anxiety are associated with higher heart rates and lower heart rate variability in response to stress [177,178]. The interaction between attachment and temperament is also seen in cardiac physiology: infants assessed in the first year of life for behavioral inhibition were at 14 and 24 months highly likely to be classified as Anxious/Ambivalent, and exhibited lower heart rate variability that could be associated with high sympathetic tone or to low vagal tone. [32,151]. However, hyperarousal, attributable to greater sympathetic activation appears to be a characteristic of anxious individuals, and particularly in children and adolescents [16,67,78,79,178,179]. Consistent with this hyper-aroused phenotype, the corticotrophin releasing hormone gene was recently linked with childhood behavioral inhibition [132,146,147], suggesting up-regulation of the hypothalamic-pituitary-adrenal axis (HPA), responsiveness to stress. Thus, an inherited predisposition for early anxious temperament and Anxious/Ambivalent attachment appear to interact to produce a constellation of characteristics involving behavioral and physiological components predictive of later childhood and adult anxiety and depression disorders [57,167].

At the other end of the spectrum of childhood disorders, aggression is a trait-like phenomenon that runs in families as well, with ample evidence for similar interactions between environmental and genetic influences [31,35,53,69,75,130]. In keeping with the notion of temperamental continuity, childhood and juvenile aggressive psychopathologies are also associated with high levels of violence in adulthood [53]. Moreover, a number of studies have shown associations between childhood aggression and attention-deficit-hyperactivity symptoms, and suggest that the two characteristics combined are more likely to produce lifelong aggressive disorders than either one alone [18,34,36]

As with inhibited temperament, insecure, Avoidant attachment behavior is associated with childhood psychopathology. Children who at early ages (12 -24 months) are categorized as showing Avoidant or Avoidant/Disorganized attachment are also more likely to manifest an *uninhibited* temperament, more aggressive and disruptive behaviors in later childhood, and higher-than-normal activity [13,31]. These children appear to be more vulnerable as well to psychosocial environments that increase the risk for child conduct disorder and adult antisocial behavior [34,69].

Finally, young children showing uninhibited temperament and Avoidant attachment exhibit higher aggression scores and higher respiratory sinus arrhythmia, indicative of reduced parasympathetic control of heart rate [4,31]. Similar relationships have been shown between autonomic activity and a history of aggression in older children [4,31,135]. In studies of 7-11-year-old boys and in aggressive adolescents, heart rate variability was found to be inversely related to hostility and aggression, consistent with reduced parasympathetic (vagal) antagonism to increases in heart rate [121,122]. It has long been known that the same relationship exists between behavior and autonomic control of heart rate and of blood pressure in hostile/aggressive adults (so-called “Type A” personalities): this mode of reduced vagal control of heart rate and blood pressure is a significant predictor of cardiovascular disease [141,142]. In summary, developmental studies have established that early aggression and reduced parasympathetic autonomic control of heart rate are markers for adult antisocial behavior and cardiovascular disease.

Although a great deal of correlational evidence points to inherited links between pathological affective regulation and physiological functioning, the conclusions drawn can only be inferential because human studies cannot examine underlying neurobiological mechanisms accounting for these associations. Animal studies, on the other hand, afford an opportunity to study the origins of these fundamental relationships. Even more interesting from a developmental perspective is the possibility of uncovering genetic processes that underlie predispositions for constellations of affective regulation, cognition, behavior, and biological processes (so-called “endophenotypes” [66]) that we call “temperament” across the lifespan. The selective breeding study to be described is one attempt to examine these relationships.

Selective breeding for high and low rates of infant vocalization responses to maternal separation

As with other infant mammals, infant rodents (pups) cry initially when separated from mothers (dams) and littermates; these cries peak at 45 kHz and are therefore called ultrasonic vocalizations or USV. Rat and mouse pups, like other mammalian species, also experience maternal separation as a stressful event, producing increased autonomic nervous system activity and cardiovascular changes, activation of the hypothalamic-pituitary-adrenal (HPA) axis, and a ramping up of noradrenergic and opioid system activity [21,72,73,76]. Thus, in response to the threat posed by separation, USV appears to be a behavioral component of an overall coordinated defensive system in rodent pups to [76,77].

Over several decades, infant rat pup USV responses to maternal separation have been well characterized pharmacologically, and studies are generally in agreement that these ultrasonic cries represent an anxiety-like state [25,77]. The question has always been whether USV can be used as a model system for infant separation responses, as described by Bowlby [22] and others. Notwithstanding caveats addressed to whether or not rats experience so complex a construct as attachment, the behavioral and physiological profiles evidenced by infant rodents when separated from dams and littermates show a great deal of face and construct validity with other mammalian/human separation responses.

Our interest in USV as a marker for early affective regulation of attachment prompted us to embark on a selective breeding project that would test the hypothesis that rate of infant USV in response to separation was heritable [24]. Behavior-genetic studies in mice had established that the propensity for high or low rates of USV is a heritable trait based on significant dominance and additive components, as well as interactions between genes [70,129]. Since no previous studies had addressed whether the behavior was itself a marker for more widespread and permanent predispositions, a second goal of this project was to determine whether rate of infant USV in response to separation was a component of a heritable temperamental trait expressed throughout life (24).

We therefore selectively bred for two lines of rats (High and Low USV lines) for extreme rates of USV in response to maternal separation in infancy. Figure 1 shows rates of USV in P10 pups for 2 minutes over 20 generations. As shown, rates of USV in High and Low USV line pups diverged dramatically from the randomly-bred control line (Random USV line) and each other, indicative of major gene effects [25,29]. With respect to the second goal, the High and Low USV lines have shown widespread and distinctly different changes in systems mediating affective regulation from infancy to adulthood consistent with both early temperament and attachment styles [24].

Weight differences at birth

An unexpected effect of selection was that Low line birth weights have been significantly lower than both High and Random line weights since the 14th (S4) generation of breeding (Figure 2A). Line differences in mean litter weights at birth have remained stable and were still highly significantly different through Generation 28 (Figure 2B). However, by weaning Low line weights are not different from the High and Random lines for either sex, into adulthood [24]. The genetic and/or prenatal mechanisms underlying this long-term reduction in Low line birth weight are as yet unknown. Low line fetuses may be genetically programmed for smaller size, or the Low line maternal uterine environment may be somehow unfavorable for growth and development of Low line fetuses. That this may result in postnatal functional changes is suggested by data in which Low line juveniles show deficits in DA-mediated behaviors (see below) that parallel deficits in animals subjected to prenatal malnutrition resulting in low birth weights [6, 80, 112, 171-173].

One conclusion arising from these data is that some of the effects of selection for low or high infant USV may be epigenetically mediated [e.g., 120], perhaps via perinatal maternal factors [58]. In the 13th generation (S13), we examined the possibility of postnatal maternal effects in the generational transmission of isolation-induced USV and behavior at P10 days. Pups in the S13 generation were cross-fostered within 48 hrs of birth between dams of the two lines (Low-pups to High-dams and High-pups to Low-dams) [30]. To control for fostering effects, other groups of pups were fostered to dams within their own lines (in-fostered). Additional (population) control data were obtained from the entire 13th generation of the selectively bred lines. P10 USV rates of cross-fostered pups in each line were almost exactly the same as rates of in-fostered pups of the same line, indicating no postnatal maternal effects on the High and Low infant USV phenotypes. However, High USV line pups cross-fostered to Low USV line dams weighed significantly less than High line in-fostered pups at P10, consistent with the idea that pre- and/or postnatal maternal-pup factors were influencing postnatal weight in the Low line. Thus, although the results provide no evidence for a postnatal maternal contribution to P10 USV phenotype, as suggested by the weight data, this limited study does not rule out either pre- or postnatal or perinatal effects on other measures of function, either in the short- or long-term.

Early differences in monoamine systems

A number of neurotransmitters have been implicated in the modulation of infant rat isolation-induced USV and in adult rat anxiety-related behaviors. Among these, influences of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) systems have been most systematically observed in rat pups [25,76]. Because High and Low line rat pups show such extraordinary extremes of USV to isolation at P10, changes in at least one of these systems seemed likely. Therefore, we examined line differences in levels and indices of activity in the three monoamine systems [26].

We found widespread changes in DA and 5-HT metabolism throughout cortico- and meso-limbic structures in both lines (e.g., Figures 3 – 4). These differences were not state-dependent, in that levels of un-manipulated pups were not different from pups exposed to 2 minutes of isolation, suggesting permanent regulatory changes in DA and 5-HT systems, in parallel with or perhaps underlying high and low infant USV rates. No changes were found in NE in any brain structures.

As shown in Figure 3A, in the anterior cingulate cortex, compared to Random line pups, in Low line levels of the DA metabolite DOPAC were significantly higher, indicative of significantly greater DA metabolism; this difference is also depicted as a higher DOPAC/DA ratio (Figure 3B). The cingulate cortex has been shown to play a central role in the production of distress vocalizations [94]; to integrate afferent and efferent sensory information [e.g. 176], and to regulate the intensity of affective expression to pain in humans and other mammals [88]. In the periaqueductal gray (PAG) levels of homovanillic acid (HVA), another DA metabolite, were significantly higher in Low and High than in Random line pups, again suggesting higher rates of DA metabolism in this structure as well (Figure 3C). The PAG is involved in the integration of affective and sensory information flowing from frontal and medial structures, and its columnar structures are dedicated to the expression of integrated, modality-specific functions, including cardiovascular, respiratory, motor and vocal responses [91, 165, 180].

Figure 4A shows levels of serotonin (5-HT) and its metabolite, 5-HIAA in the bed nucleus of the stria terminalis (BNST) in the three lines. In this structure both High and Low lines showed higher levels of both 5-HT and 5-HIAA, suggesting higher levels of production and neurotransmission of 5-HT. The BNST is involved in modulating anxiety to both learned and unlearned stimuli, and in corticotropin-releasing hormone signaling in stress-induced cardiac activation [44, 109]. In the striatum (globus pallidus and putamen) levels of 5-HIAA were higher in High line pups (Figure 4B), signifying greater metabolic activity in this structure involved in the coordination of species-typical motor activity [118].

The significance of these global changes in monoamine function early in life is unclear without additional information from other methods, although they clearly point toward alterations in DA- and 5-HT systems in structures that are not occurring in Random line pups. Nor is it clear that levels of activity of these monoamines have the same significance in the early postnatal period as in adulthood, since at this time both systems are undergoing rapid development in cortico- and mesolimbic structures [43,47,62,63,120,156,181, and see below]. Unlike adulthood, in which 5-HT generally functions as an inhibitory modulator, at this stage of life 5-HT increases neuronal activity in its role as a neurodevelopmental factor in cell growth and differentiation [11,12,93,119,182]. Similarly, the number and firing rates of spontaneously active DA neurons change during maturation of frontal and pre-limbic systems from 2 to 4 weeks of age [39,101,91]. Moreover, a variety of perinatal experiences produce discontinuities in the development and activity of these systems so as to permanently alter monoamine functioning in target areas [7,38,38]. Thus, it seems likely that the extensive changes in DA

and 5-HT systems in High, and especially in Low line pups are predictive of functional alterations later in life.

Social play in the juvenile period

Play is one of the earliest social behaviors that preweanling rats engage in that are not oriented toward the dam. In rats, play begins at about P18, shortly after eye opening and with independent feeding, and peaks during the periadolescent period between 30 and 40 days of age [98,113,157,162]. As in other mammals, play in rats is thought to have paralleled the evolution of the prefrontal cortex [46,51,117]. Consistent with this view, rat play is comprised of complex sets of behavioral interactions that have been implicated in shaping the development of social and cognitive skills in adulthood characterized by cognitive flexibility, such as turn-taking or the ability to switch cognitive strategies [48-50,117].

A variety of perinatal conditions affect play behaviors in juvenile rats. Adult rats that have suffered perinatal malnutrition, or have been reared without access to play partners show similar patterns of social reactivity and inability to communicate socially [171-173]; and malnourished animals are more aggressive as adults than normally nourished controls [92, 158,172]. Social play in adult and juvenile rats also varies by rat strain, indicating underlying genetic variation in levels of playfulness [139,140,158].

Since selection for an early, social, affective-related behavior such as USV may also have influenced the development of social behavior, in a recent study [28] we investigated whether selective breeding for high and low USV rates affected common indices of juvenile play behavior, and ultrasonic vocalizations associated with play (50 kHz calls) [81]. High, Low and Random line juveniles were isolated overnight and allowed to play briefly on each of three subsequent days, in same-line, same-sex sibling pairs. Interactions were observed over 10 minutes and frequencies of play behaviors, including nape contacts, pins and 50 kHz USVs were counted by pair [5,6,28]. Definitions of these behaviors are given in detail in [28].

Only play behaviors were reduced in the selected lines, ruling out global deficits in behavior, locomotor abilities or differences in arousal. High line juveniles had higher latencies to pin and lower levels of pinning (Figure 5A) and 50 kHz USVs (Figure 5B), but only in the first of the three play sessions (Play Session 1). Since 50 kHz USV is a reliable marker of positive affect in rats [81, 82], we interpreted the depression of pinning and USVs in High line on the first day to signify a negative affective state associated with initial conditions of exposure to a play partner. In contrast, nape contacts in High play pairs were uniformly low across all three play sessions (Figure 5C), suggesting ongoing reactivity to social isolation between play bouts, or that this specific behavior deficit may be an enduring characteristic of the High line, or both. Thus, it seemed reasonable to conclude that play deficits in High line juveniles were a function of behavioral inhibition or anxiety, consistent with their infant phenotype [25]. The results are also consistent with studies of 5-HT or GABA influences on regulation of affective states affecting social behavior [65, 118, 154].

In contrast, Low line juveniles were deficient in all play behaviors: both in terms of latencies to engage in play, and in frequencies of nape contact, pinning and rates of 50 kHz USV. Hypotheses for Low line play deficits can be posited: for example that Low line juveniles lack motivation to initiate and engage in play and therefore are affectively compromised. Alternatively, Low line juveniles may be motivated, but unable to “read” social signals intrinsic to play, with the result that juvenile play behavior is significantly reduced, without affecting pro-social behaviors like social investigation [54].

Similar patterns are evident in juveniles subjected to social isolation, and in juveniles lacking prefrontal cortex or striatum [50,114,118]. Global deficits in social play such as these are

thought to result from reductions in dopamine (DA) levels and functioning [10,54,157]. Stimulation of DA activity increases play, probably through the combined action of pre- and postsynaptic dopamine D2 receptors [162]. Various forms of perinatal insults are known to affect DA functioning [15,64,105-107,110,153,154]. These conditions produce regionally specific effects on DA levels and DA activity (measured as DA metabolite turnover) in prefrontal cortex, hippocampus, hypothalamus and striatum [e.g., 68,80,85]. Thus, one likely mechanism for deficits in Low line play is via dysfunction in structures in systems mediating play. At another level, deficits in play may be associated with putative deficits in DA system function, with or without links to selected genes affecting USV.

Measurement of monoamine metabolism in juvenile brains after play are currently underway to address the question of developmental continuity in DA and 5-HT activity and function from infancy, and to correlate monoamine levels with differences in play between the lines. Future studies using more sensitive, fine-grained behavioral analyses will also aid in resolving questions about the nature of behavioral deficits affecting patterns of play in both lines, in conjunction with pharmacological interventions specifically targeting the DA systems.

Developmental continuity in adult affective regulation

If separation-induced USV rates are an indicator of an infant anxiety-like state, does it follow that extremes of infant USV rates predict anxiety-like behavior in adulthood? Human studies examining samples of children expressing childhood inhibition and adult anxiety/depression disorders suggest that this may be the case. On the other hand, expressions of infantile states are not necessarily predictive of lifelong behavioral traits. Infantile isolation-induced USV may, for example, be an instance of a class of purely infantile behaviors or “ontogenetic adaptations” specific to the ecological niche occupied by infants in the postnatal period [170].

To test this question of continuity, High, Low and Random line adults of both sexes were examined in a variety of standardized laboratory tests measuring anxiety and depression in rodents [184]. In a variation of the open field, to test fear of open spaces, High and Low line males and females were placed in a small cylinder in the open field, and the latencies at which they emerged from the cylinder were recorded. As shown in Figure 6, latencies for High line males were significantly higher than for Low line males. Similar results were seen in females (data not shown). High line adults of both sexes also traversed fewer inner squares than Lows, another indication of higher anxiety-like state.

Figures 7A and B show two replications across generations of line differences in High versus Low adult males in the lengths of time spent floating, one of several behaviors measured in the Porsolt Swim. The Porsolt Swim is considered a reasonable model for depression-like behavior in rodents, since behavioral measures such as floating respond primarily to antidepressant, but not anxiolytic action [65]. In both generations High line animals of both sexes (female data not shown) spent more time floating than Lows, indicating a particularly reliable difference between the lines [137, 184]. Interestingly, differences between the lines in the Porsolt Swim are more robust than some anxiety measures like the plus maze, suggesting that the High infant USV phenotype may be more predictive of adult depression-like states than anxiety [24]. In this respect, the expression of High line infant and adult phenotypes resemble human family-genetic studies, in that child anxiety phenotypes tend to resolve into depressive disorders in adulthood [168].

In the same study, we measured levels of the neurosteroid, allopregnanolone (3-hydroxy-5-pregnan-20-one; $3\alpha,5\alpha$ -THP), a reduced metabolite of progesterone. Allopregnanolone, a positive modulator of the GABA_A receptor, reduces anxiety behavior in adult rats in a variety

of paradigms, including rat pup isolation calls [20,23,33,59-61,127,164,183]. Moreover, allopregnanolone modulates and is itself modulated by the 5-HT system and has been shown to alter depressive states via 5-HT mechanisms [68,161]. Because neurosteroids appear to be intimately involved in stress responses [8,9], we thought it possible that the adult behavioral differences observed between the Low and High USV lines might be due to selected differences in allopregnanolone-modulated receptor systems.

Allopregnanolone levels were measured in hippocampus and amygdala tissue in the same animals tested for behavior, and were significantly higher in Low line male (Figure 8A) and female (Figure 8B) rats than High line rats, consistent with its acute anti-depressant and anti-anxiety effects in rodents [19, 20, 23]. In both selected lines allopregnanolone levels fluctuated with the estrus cycle in females, so that higher allopregnanolone levels and reduced anxiety occurred during estrus when estrogen levels are highest; these were reversed during diestrus when estrogen levels are low (Figure 8B). It is notable that comparable changes in allopregnanolone levels have been implicated in Pre-Menstrual Syndrome disorder in women, and mechanisms underlying the relationships between fluctuations of mood with changes in ovarian hormone levels have been shown to be mediated by effects in the brain of allopregnanolone withdrawal on GABA_A subunit conformation in rodent models [145, 155]. Higher allopregnanolone levels in Low line adults in both sexes suggests the ability to mount greater synthesis of allopregnanolone in brain areas mediating stress in the Low line. Alternatively, Lows may have higher baseline endogenous allopregnanolone or of its precursor, progesterone, and thus greater reserves to draw from during stress. Either process would promote enhanced functioning during stress.

Autonomic nervous system (ANS) regulation of heart rate

Selection for infant USV has also produced distinct modes of cardiac responsiveness to stress. In a study of 18-day-old juveniles we found that compared to the Random line both the High and Low USV lines exhibit enhanced cardiac reactivity to stress [27]. Figure 9 shows heart rate changes at Baseline (Home cage), Isolation (Novel cage) and Recovery (Home cage), in 2-min epochs, in P18 juveniles that were taken from the home cage and placed individually into a novel environment, then returned to the home cage. Though neither line showed heart rate differences at baseline from Random, High line juvenile heart rates were significantly higher than Random line heart rates during isolation in the novel environment, consistent with their anxiety phenotype in infancy and in adulthood. In contrast to their apparent lack of anxiety in behavioral tests, however, juvenile Low line heart rates were significantly higher than both Randoms and Highs, indicative of even greater reactivity to novelty. Note that neither High nor Low line heart rates returned to baseline during recovery in the home cage, indicating continuing reactivity to the stressor in both lines, compared to the drop in Random line heart rates.

In determining relative influences of sympathetic or parasympathetic systems on heart rates in animals, each branch can be blocked directly, pharmacologically, revealing the influence of the other in its absence. Since the sympathetic nervous system *increases* heart rate via adrenergic influences on the heart, in this study adrenergic action was blocked with atenolol, a cardio-specific beta-adrenergic antagonist used to block the influence of sympathetic activity (and sympathoadrenal effects). In contrast, the parasympathetic nervous system *reduces* heart rates via cholinergic vagus nerve activation, therefore atropine, a quaternized muscarinic antagonist with minimal central effects, was used to block parasympathetic activity. Pharmacological blockade revealed that in High line juveniles higher heart rates during isolation stress were the result of greater sympathetic *acceleratory* influence on heart rates. These findings are consistent with populations of children prone to anxiety [30]. The even higher heart rates of Low line juveniles during isolation stress were clearly due to greater

parasympathetic *withdrawal*, largely eliminating the only braking influence on rising heart rates; however, some sympathetic influences increasing Low line heart rates beyond controls could not be ruled out in this study.

Adult males demonstrated continuity with juvenile heart rate responses during 30 minutes of restraint stress [137]. As shown in Figure 10A, in the first 10 minutes heart rates in High and Low adult males were significantly higher than Random line males; but as animals habituated to restraint, High and Random heart rates declined similarly over time. In contrast, Low line males maintained their high heart rates for the entire 30 minutes. High-frequency variability (r-MSSD [108]) was lower in the Low line males throughout the 30-minute period (Figure 10B), indicative of lower parasympathetic (vagal) restraint on heart rate. Post hoc tests demonstrated that high-frequency variability in Lows was low than both Highs and Random males, consistent with a lack parasympathetic restraint on heart rate in Low line rats. At the same time, blood pressure variability was greater in Low line adult males, a condition that predicts later elevated blood pressure [142].

Although only suggestive, early life alterations in DA and 5-HT transmission patterns in the PAG and the BNST may have had considerable effects on fine-tuning juvenile and adult cardioregulatory processes. Both of these central structures are involved in autonomic and cardiovascular responsiveness, and particularly the PAG is the final afferently-regulated medial structure before more automatic processing occurs in brain stem [180].

However striking, this finding is not consistent with an earlier study of adult High, Random and Low line males heart rates tested under the same conditions of restraint. In that study, both High and Low males maintained higher heart rates for the entire 30 min, suggesting that both selected lines were hyper-reactive to the confines of restraint [24]. The reasons for the discrepancy between the two studies may be due to a variety of factors, among them handling experience, temperature, to differences in sleep state, or as a consequence of struggling more during restraint. To control for extraneous variables on heart rate, currently we are studying heart rate differences in adult males under anesthesia, again using atenolol and atropine to blockade sympathetic and parasympathetic nervous systems influences on cardiac and blood pressure function.

Although higher sympathetic activation of heart rate is common in children and adults suffering from anxiety disorders (e.g., [67,179]) greater parasympathetic withdrawal, sometimes combined with higher sympathetic regulation of heart rate is characteristic of patients suffering from depressive disorders [1,2159,160,178]. Thus it is possible that High line adults will exhibit either the same sympathetic over-activity that characterizes High line juveniles, or lower parasympathetic activity, or both, in line with their anxious/depressed lifetime profile. Conversely, Low line cardiovascular functioning is expected to be characterized primarily by parasympathetic under-activity, consistent with their low anxiety profile, and paralleling human populations exhibiting lifelong aggression/hostility [141,142].

Low Line male – male aggression

As noted above, another potential component of a Low line model suggested by autonomic and behavioral data would be elevated aggressive behavior. To test this hypothesis we paired Low line adult males with novel partners of the same line and compared them to comparably-treated males in the Random line, in a novel but neutral, non-threatening environment (Social Interaction test [55]). Since rats are not generally aggressive in this context, males of both lines were previously socially isolated in home cages for 2 weeks, a procedure that increases behavioral reactivity and aggression in about 20 – 30% of rats, depending upon strain (e.g., [175]). As shown in Table 1, about a third of Random line rats responded to this treatment with

increases in aggression, that is, within the range observed in other outbred rat strains. In contrast, more than 70% of Low line pairs treated in this way engaged in fights and exhibited freezing behavior associated with fighting. These results suggest that higher than normal aggression in male-male encounters after social isolation is indeed highly characteristic of the Low line.

In rats, social isolation decreases allopregnanolone levels by 60-70% in cerebral cortex, hippocampus and blood plasma [136]. Decreased frontal cortex allopregnanolone is associated with reductions in 5-alpha-reductase-I, the enzyme involved in the rate-limiting step in the biosynthesis of allopregnanolone from progesterone [99]. This isolation-induced down-regulation can be reversed by applications of low concentrations of allopregnanolone [8,9]. However, when exposed to an acute stressor like footshock, isolated animals show significant *increases* in brain and plasma concentrations of allopregnanolone over baseline and over those of socially-housed controls [8,9]. Pretreatment before social interaction with diazepam, a GABA_A modulator at the benzodiazepine receptor site, reduces aggressive behavior in isolated rats in a dose-response fashion [40,175]. Thus, in rodents social isolation produces baseline reduction in allopregnanolone and hence GABA_A function, with an apparent rebound in allopregnanolone levels in the face of stress that may mediate increases in aggressive behavior. Other studies have shown that lower levels of GABA are found in mice and rats that are genetically predisposed to aggression or exhibit more aggression under experimental manipulation, suggesting that some individuals are more at risk [100].

Given that brain allopregnanolone levels are higher in Low line animals in response to a stressor [184], one possible scenario underlying higher aggression in socially isolated Low line males is that brain allopregnanolone levels are reduced by social isolation below that of Random line control males. In response to subsequent interaction with strange conspecifics Low line brain allopregnanolone overshoots comparable rises seen in similarly isolated Random males, essentially disinhibiting aggression via GABA_A receptor mechanisms [9,19].

Emotional Regulation, Attachment and Coping Styles

It appears then, that selective breeding for high and low rates of vocalization in response to maternal separation has produced two distinct temperamental styles that include alterations in behavioral, physiological, and neurochemical characteristics. The integrity of these personal styles are continuous across the life span, and express themselves as adult phenotypes comparable to those seen in human populations. In humans and in the selected lines, the origins of behavioral predispositions appear to lie early in development, and consist of unique separation anxiety and attachment responses. Concomitantly, system-wide alterations of physiology are associated with and are part of these overall phenotypes.

In this respect, the High and Low lines share traits distinguishing other animal models selected for “high” or “low” values of systems associated with anxiety/depression or of aggression/impulsivity (e.g., Wistar-Kyoto, Spontaneously Hypertensive Rats; High Anxiety Behavior, Low Anxiety Behavior rats; [111,126,131]). Such clusters of behavioral and physiological traits (endophenotypes [66]) are observed in other species including humans, that mark “passive” and “active” coping styles in response to environmental demands [14,45,48,74,86,143,144;150]. Recently, this notion has been placed in the context of Darwinian evolutionary theory [87], in which species-wide selection pressures (ultimate and proximate factors) favor preservation of genes modulating endophenotypes associated with each coping style [3,149]. In a given population, the function of these processes is to maintain organismic stability (allostasis) in adaptation to divergent or extreme environmental demands. Each coping style may be of benefit in a specific type of environment, but when expressed as extreme variants (e.g., [163]), may ultimately lead to overwhelming, but different classes of psychological and

physiological costs (allostatic load), to chronic or overwhelming stress [87]. In that case, dysregulation of affective states, decreased neurogenesis and increased cell death, metabolic disorders, cardiovascular syndromes and altered inflammatory and immune responses occur that lead to illnesses that are specific to each style [87].

It is not clear how closely assemblages of endophenotypes displayed by the Low and High lines will conform to the dimensions of behavior and physiology attributed to “active” and “passive” coping styles. It would not be surprising if High and Low lines showed variation in the expression of endophenotypes associated with anxiety or aggression temperaments compared to other such populations. Indeed, it would be surprising if there were *not* variations in endophenotypes in populations produced by a variety of natural conditions and manipulations, including mouse genetic knock-out technology (e.g., [56,65,71]). The High and Low USV lines are the first to demonstrate that these coping styles arise from and are continuous with early life temperamental differences. Moreover, they are the first genetic animal model implicating long-term associations of each style with early attachment responses. As such, they present a potentially powerful model for examining neurobiological gene-environment interactions in the development of life-long affective regulation.

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Figure 1.

Mean ($\pm$ SEM) number of USV in P10 pups for 2 minutes (y-axis) over 20 generations (x-axis). Low and High USV lines diverged significantly in rates of USV from the Randomly-bred control line in the S1 and S3 generations respectively. Data based on litter means. USV was transduced by a bat detector (UltraSound Advice, London) set on broadband mode (10 – 100 kHz), and therefore the frequencies of USVs selected for in the lines were theoretically anywhere within this range. From Brunelli, 2005.

Figure 2.

A. Mean ($\pm$ SEM) birth weights (grams) High, Low and Random lines, across the 14th through 21st generations: Line $F(2,1196) = 44.634$, $p = 0.000$; Generation: $F(6, 1196) = 6.277$, $p = 0.000$; Generation *Line: $(12,1196) = 2.146$, $p = 0.012$. Data based on litter means (unpublished data).

B. Mean ($\pm$ SEM) birth weights averaged over 22nd – 28th selected generations, showing stable differences between Low and High/Random litters Line $F(2,49) = 7.175$, $p = 0.002$. Data based on litter means (unpublished data).

Cingulate Cortex

Figure 3.

A. Mean (±SEM) DA and DOPAC levels in accessory cingulate cortex (ACC), measured as picograms per milligram of tissue (n=12 pups All ps < .01 per line). Low line DOPAC significantly greater than High and Random lines). From Brunelli & Kehoe, 2005. P10 pups from 6 High, 6 Low and 6 Random line litters were rapidly decapitated and the brains quickly removed, dissected and frozen in dry ice after: 1) 10-min isolation at ambient temperature; or 2) removal directly from the litter pile in the home cage. Two pups per litter per group were used for each brain sample. Dissected brains were frozen at -80°C until monoamines were extracted and measured by means of high performance liquid chromatography.

B. DOPAC/DA ratio in ACC). All ps < .01. Low line significantly greater than High and Random lines. From Brunelli & Kehoe, 2005.

C. Mean (±SEM) DA, DOPAC and HVA levels in periaqueductal gray (PAG), measured as picograms per milligram of tissue. All ps < .01. High and Low line HVA significantly greater than Random. From Brunelli & Kehoe, 2005.

Figure 4.

A. Mean ($\pm$ SEM) levels of 5-HT (picograms per milligram of tissue) and 5-HT turnover measured as levels of 5-HIAA (picograms per milligram of tissue) in the bed nucleus of the stria terminalis (BNST). All p s < .01. High and Low line levels of 5-HT and 5-HIAA significantly greater than Random line. From Brunelli & Kehoe, 2005.

B. Mean ($\pm$ SEM) levels of 5-HT (picograms per milligram of tissue) and 5-HT turnover in striatum (caudate + putamen) and nucleus accumbens (NA). High line levels of 5-HIAA significantly greater than Low and Random lines.

Figure 5.

A. Mean ($\pm$ SEM) number of pins in 10 minutes of play during three successive play sessions in High, Low and Random juvenile sibling pairs. Low line significantly less than Random line. Male and female pairs are shown separately for each line. Frequencies shown are for sibling pairs, which were scored as one unit. From Brunelli, et al., 2006.

B. Mean ($\pm$ SEM) number of 55 kHz vocalizations emitted by High, Low and Random sibling pairs in 10 minutes during three successive play sessions. Low line significantly less than Random line. From Brunelli, et al., 2006.

C. Mean ($\pm$ SEM) number of nape contacts in 10 minutes of play during three successive play sessions (one per day over three days), in High, Low and Random line sibling pairs. Low and High lines significantly less than Random line. From Brunelli, et al., 2006.

Figure 6.
Mean ($\pm$ SEM) latency to emerge from a cylinder into an open field by males High and Low USV lines. From Zimmerberg, et al., 2005.

Figure 7.

A. (left) Mean ($\pm$ SEM) duration immobile (floating) in the Porsolt Swim by males in the High, Low and Random lines in the 15th generation (S15); and **B. (right)** in High and Low line males in the 19th (S19) generation of selection. From Shair et al., 2000; and Zimmerberg, et al., 2005.

Figure 8.

Mean ($\pm$ SEM) concentrations in ng/g tissue of allopregnanolone (3-,5-THP) in amygdala/hippocampal tissue of males (A), and proestrus females and diestrus females (B) from selectively bred High and Low USV lines (significant main effects of line and hormone status, n 's = 13–17 subjects per bar). From Zimmerberg, et al., 2005.

Figure 9.

Mean ($\pm$ SEM) heart rates (HRs, in beats per minute [bpm]) of Postnatal Day (P) 18 control pups in the High-USV, Low-USV, and Random-USV lines, from Baseline in the home cage (Home Cage, left) through Isolation in a novel cage (Novel Cage, middle), to Recovery back in the home cage (Home Cage, right). From Brunelli, et al., 2002.

Figure 10.

A. Mean ($\pm$ SEM) adult male heart rates over 30-min of restraint at 10-min intervals. Significant Line effect ($p=.013$), which post hocs demonstrate are due to Low > Random line heart rates. Significant Line $\times$ Epoch effect ($p = .05$) due to Low > Random/High line heart rates at the 30 minute epoch. From Shair et al., 2000.

B.) Mean ($\pm$ SEM) adult male r-MSSD ($\pm$ standard error), calculated as root mean square of successive beat-to-beat variability (an index of high frequency variability) [Murphy, et al., 1991; 99] over 30-min of restraint at 10-min intervals. Significant Line $\times$ Epoch effect ($p = .05$) due to Low < Random/High line at the 30 minute epoch. From Shair et al., 2000.

Table 1
Percent (number) of Low-Low and Random-Random adult male pairs fighting or freezing (unpublished data).

	Fight *	NO	Freeze **	YES	NO
Low %	70.8	29.2	60.8	39.2	100
n =	(17)	(7)	(15)	(9)	(24)
Random %	35.3	64.7	18	82	100
n =	(6)	(11)	(3)	(14)	(17)
Total N =		41			41

* Pearson Chi-square=5.103,df=1,p=0.024;

** Pearson Chi-square=8.129,df=1,p=0.004