ANIMAL MODELS AND ENDOPHENOTYPES OF ANXIETY AND STRESS DISORDERS

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ANIMAL MODELS OF PSYCHIATRIC ILLNESS

Animal Models: Types Of Validity

An important criterion for developing animal models to study psychopathology involves establishing the validity of the model as a true representation of the process being studied. Generally, three types of validity are applied to animal models: face validity, construct validity, and predictive validity (1-3). Face validity refers to the outward similarity in appearance between the model and the illness. Construct validity, on the other hand, does not exclusively involve outward tangible signs of the modeled illness. Rather, it refers to the internal mechanism or state that underlies the illness. Finally, predictive validity refers to the ability of the animal model to identify therapeutic treatments for the illness. It should be noted that the different types of validity can be independent of each other; an animal model can possess predictive and construct validity without possessing face validity. Ideally, an animal model should possess both construct and predictive validity so that it may be used to understand the mechanisms and etiology of the disorder and also to identify promising treatments for the disorder.

Endophenotype Approach

Species differences in the manifestation of a particular internal state can cloud the usefulness of face validity in animal models. In addition, when considering a complex psychiatric illness, it is likely that several different symptom clusters contribute to the final pathologic condition; these different sets of symptoms may have different underlying substrates and thus may be ameliorated by different treatments. There-

fore, it is difficult to come up with an animal model for an illness that meets the aforementioned criteria and also models the pathologic syndrome in its entirety. An alternative approach that has been used involves the modeling of discrete symptom clusters and physiologic alterations rather than the whole syndrome, with the assumption that what causes the symptoms contributes mechanistically to the illness. This general approach has involved the use of endophenotypes that may be related to a particular psychiatric disorder. The term *endophenotype* refers to a set of behavioral and/or physiologic characteristics that accompany a basic process that is altered in relation to the illness that is being studied (4). It is important to note that this more narrowly defined endophenotype approach does not necessarily have to capture specific symptoms that are a part of the clinical diagnosis, but rather may focus on a core process or function that is abnormal in the clinical population under study and that is thought to be related to the manifestation of the illness. For example, in the case of anxiety-related disorders, investigators have focused on studying the genetic, physiologic, and neurochemical correlates of fearful or anxious endophenotypes because a core aspect of anxiety-related disorders involves the aberrant expression of fearful responses to neutral or mildly stressful contexts (5). Thus, by identifying animals that display fearful endophenotypes, it is possible to study the neural substrates that contribute to this basic process that may underlie the development and expression of anxiety-related psychopathology.

Using endophenotypes that are based on core and basic processes rather than the entire illness offers certain advantages. Because the whole illness is not being modeled, the endophenotype approach affords greater possibility for construct and predictive validity in the model, and can incorporate species-specific manifestations of the core process being modeled. This approach may also make screening for genetic abnormalities associated with the disorder more fruitful, because the genetic factors associated with a very discrete

process (which could be mediated by a small number of genes) rather than an entire syndrome (which is likely caused by a complex set of interactions between multiple genes) is being studied (4,6). Moreover, heterogeneity within a diagnostic category could potentially dilute the strength of a sample population (i.e., not all patients with anxiety disorders are identical in their clinical presentation), and diminish the chances of identifying genes that contribute to the illness. Ideally, one might be able to generate several different endophenotypes for a particular disorder, and then study the genetic underpinnings of each of these separate core processes in order to identify a set of genes that might be implicated in that particular disorder. The definition and use of endophenotypes in animal models of psychiatric illness is a developing area. This chapter presents some promising candidates of animal models of fearful and anxious endophenotypes, and outlines some of the preliminary genetic factors that have been identified to contribute to the manifestation of these endophenotypes.

PUTATIVE ANIMAL ENDOPHENOTYPES OF STRESS AND ANXIETY: STUDIES OF FEARFUL TEMPERAMENT

Defensive Behaviors

In an attempt to understand the basic neural mechanisms underlying psychiatric conditions involving fear and anxiety, several groups have focused on identifying the neural substrates of defensive behaviors in animals. Defensive behaviors are exhibited by a wide array of species including rats, nonhuman primates, and humans in response to perceived threats from the environment, and are essential components of an organism's behavioral repertoire that ensure its protection and survival. Because organisms display defensive behaviors in reaction to threat, it is thought that the aberrant expression of defensive behaviors may represent a good example of a fearful endophenotype that would have relevance to stress and anxiety-related disorders. Although the specific behavioral responses that compose defensive behaviors are dependent on the environmental context and vary from species to species, a common element that unites this cross-species phenomenon is that defensive behaviors represent an organism's behavioral response to fear. Because defensive behaviors are expressed in response to an immediate threat, they characteristically supersede and interrupt the expression of other normal homeostatic behaviors such as feeding and reproduction that may be ongoing at the time of the perceived threat (7-11). One defensive response pattern expressed by many species is to inhibit all body movements and assume an immobile or freezing posture. This phenomenon of behavioral inhibition is effective in preventing detection and attack by predators (12,13), and may have special relevance for understanding psychopathology.

In nonhuman primates, defensive behaviors are com-

posed of a constellation of responses that include vocalizations, freezing, fleeing, or defensive hostility and aggression. The particular set of responses that is emitted depends on, among other variables, the nature of the perceived threat (14,15). Studies of defensive behaviors in rhesus monkeys may provide valuable information that could aid in the understanding of fear and anxiety-related psychopathology in humans, because extreme fearful or defensive responses occur in dispositionally fearful humans who have an increased risk to develop psychopathology (16).

Psychiatric illnesses such as anxiety disorders and depression might involve the aberrant expression of defensive behaviors. In other words, pathologic anxiety could be conceptualized as the inappropriate expression of defensive or fearrelated behaviors, consisting of either an exaggerated or overly fearful response to an appropriate context, or a fearful response to an inappropriate or neutral context. Although appropriate levels of defensive behaviors in response to environmental threats are adaptive and ensure survival, the overly intense or context-inappropriate display of fearrelated defensive behaviors may represent a liability that interferes with normal behavior and would likely contribute to certain forms of fear-related psychopathology. Thus, inappropriate or exaggerated expression of defensive behaviors may represent an important animal endophenotype of anxiety. An understanding of the specific neural substrates underlying the expression and regulation of defensive behaviors may therefore ultimately shed insight into the processes that become dysregulated in stress-related psychopathology. In defining animal endophenotypes relevant to anxiety, specific symptoms of a particular type of anxiety disorder are not being modeled, but rather the general phenomenon of hyperreactivity to mildly stressful stimuli is studied. The approach of modeling anxiety by studying defensive behaviors in animals has been described previously for rodent models (17,18). In the following sections, both primate and rodent analogues of stress hyperresponsiveness are described, with a particular emphasis on models of either the overly intense but context-appropriate expression of defensive behaviors or the normal but context inappropriate expression of defensive behaviors. Initially, various behavioral paradigms that have been used to measure an animal's level of defensive behavior are described, and subsequently, specific examples of fearful endophenotypes that have been identified using these tests are discussed.

Measuring Defensive Behaviors in Nonhuman Primates: Human Intruder Paradigm

One laboratory paradigm that has been developed to identify animals with fearful dispositions characterizes monkeys' fearful behavioral responses to a human intruder. In the human intruder paradigm (HIP), the monkey is placed by itself in a test cage where it remains for 30 to 40 minutes

while its behavior is recorded on videotape. A human intruder then enters the test area, representing a potential predatorial threat to the animal (14,15,19). The test session consists of three consecutive brief conditions: alone ("A," animal left alone in cage); no eye contact ("NEC," animal presented with the facial profile of a human standing 2.5 m away); stare ("ST," animal presented with a human who faces it and engages it in direct eye contact). Typically, animals respond to the A condition by increasing their levels of locomotion and by emitting frequent coo vocalizations, which have been likened to the human cry and function to signal the infant's location and facilitate maternal retrieval (20,21). The NEC condition causes a reduction in cooing and an increase in behavioral inhibition, which functions to help the monkey remain inconspicuous in the face of a predator and is often manifested as hiding behind the food bin and freezing. The ST condition elicits aggressive (openmouth threats, lunges, cage shaking, barking vocalizations) and submissive (lip smacking, fear-grimacing) behaviors that represent adaptive responses to the perceived threat of the staring experimenter. The different test conditions (A, NEC, ST) reliably elicit responses in young or adult laboratory-reared monkeys or in feral animals (14,19). Moreover, these context-specific defensive responses are not dependent on the gender of the intruder, and can also be elicited by showing the animal a videotape of the intruder (Kalin et al., unpublished data).

Behavioral Tests Used to Measure Fearful Endophenotype in Rodents

To identify fearful endophenotypes in rodents, a variety of behavioral paradigms have been employed. All of the paradigms in some manner provide an assessment of the rodent's level of defensive behavior, which is essentially thought to be an index of its level of fearfulness or anxiousness. The behavioral tests measure one of four general categories of stress-related behavior: approach-avoidance conflicts, conditioned fear, aggression, and punished responding conflicts. Detailed descriptions and protocols for these tests can be found in a recent review by File and colleagues (22).

Approach-Avoidance Conflicts

Briefly, all of the paradigms presented in this section measure the animal's ratio of approach versus avoidance behaviors by presenting a choice between an environment that is safe (usually a dark, enclosed, small space) and an environment that seems novel but risky (usually bright, wide open, large spaces). The entries into and amount of time spent in the safe environment relative to the risky environment are used as an index of the animal's stress level (an increase in exploratory behaviors toward and into the risky environment indicate a relatively low level of stress). A number of

paradigms including the *elevated plus maze* (composed of safer closed, dark arms versus riskier open bright arms), the *open field* (consisting of a darker wall-bordered peripheral portion versus a brighter open center section), a *light-dark transition box* (consisting of an exploratorium divided into two halves, one that is dark and one that is bright), and a *defensive withdrawal* apparatus (composed of a small dark chamber that is inside of a brightly lit open field) have been frequently used and validated as paradigms that are sensitive to detecting shifts in an animal's approach-avoidance—based conflict (23).

Conditioned Fear

Behavioral tests that measure conditioned fear utilize basic principles of Skinnerian conditioning. Two frequently used paradigms to assess fear conditioning are conditioned freezing and fear-potentiated startle. Conditioned freezing is evaluated using a two-step procedure. First, during the training or conditioning phase, a stressful unconditioned stimulus (UCS, such as a foot shock) that elicits freezing is paired with a neutral stimulus that subsequently becomes a conditioned stimulus (CS). On the test day, the amount of freezing in response to the CS is assessed; animals that have not undergone the CS-UCS pairing do not normally freeze when the CS is presented, but animals that have learned to associate the CS with a foot shock show marked levels of freezing simply in response to this stimulus. The CS can either be a context (i.e., the environment in which the shock is delivered) or a discrete cue (e.g., a tone or light). The level of conditioned freezing is thought to correspond to the level of fear or anxiety that the animal is experiencing due to anticipation of a threat (24). In the case of fearpotentiated startle, the unconditioned startle response to a sudden stimulus (e.g., a loud noise burst) is measured in the presence and in the absence of a CS that has been paired previously with shock. The startle response is markedly increased when the startling stimulus occurs in the presence of the CS; this relative increase in startle magnitude is quantified, and serves as an index of the level of fear (thought to be elicited by a discrete cue as the CS) or anxiety (thought to be elicited by a contextual CS) that the animal may be experiencing (25).

Aggression and Social Behavior

Aggressive behaviors are emitted as part of the behavioral repertoire an animal displays when it encounters a threatening situation. The study of defensive aggressive behaviors has been summarized and reviewed by a number of investigators (26–28). Briefly, aggressive behaviors can be studied using a *resident intruder paradigm*, in which the offensive/ agonistic responses (e.g., upright postures, attacks) of a male resident or the defensive responses (e.g., submissive posture, flight, freezing) of a male intruder are measured. Other

stress-related paradigms involve the study of affiliative behaviors and include the *social interaction* test in which approach toward and contact between two rats is measured (e.g., sniffing or grooming each other).

Punished Responding Conflict

The basic principle of punished responding tests is to present the animal with a situation in which a particular behavioral response results in both a rewarding outcome and an aversive outcome. The extent to which the animal exhibits the behavioral response during the conflict schedule is used as an index of its level of stress. For example, in the classic Geller-Seifter conflict test (29), rats are trained to press a lever for a food reward. Gradually, the bar press is also paired with a mild foot shock, and a stable rate of responding is established under the conflict schedule. Drugs can then be administered and evaluated for their ability to increase responding under the punished schedule. For example, benzodiazepines have been found to increase bar-pressing during the conflict schedule, putatively by decreasing the stress or anxiety induced by the aversive stimulus. Similarly, in the Vogel punished drinking paradigm (30), thirsty rats with access to a water bottle are periodically given mild electric shocks through the spout of the bottle; the extent to which licking is decreased is used as an index of stress.

INDIVIDUAL DIFFERENCES IN DEFENSIVE BEHAVIORS: NATURALLY OCCURRING FEARFUL ENDOPHENOTYPES

Primates

Several lines of evidence support the notion that an individual's level of defensive responding is a relatively stable trait characteristic (which in part may be derived from the nature of early postnatal maternal interactions, see below). Extreme individual differences detected early in life may be predictive of future psychopathology. For example, extremely inhibited children are at greater risk to develop anxiety and depressive disorders and are more likely to have parents that suffer from anxiety disorders (31–34). Moreover, behavioral inhibition in childhood (based on retrospective self-reports) is highly associated with anxiety in adulthood (35). Some of the physiologic correlates that have been observed in extremely inhibited children are elevated levels of the stressrelated hormone cortisol (36) and greater sympathetic nervous system activity (37). In nonhuman primates, individual differences in defensive behaviors have been studied in an attempt to elucidate the neuroendocrine and neurobiological concomitants of extreme behavioral inhibition and to characterize a primate analogue of an anxiety-related endophenotype.

Marked individual differences among rhesus monkeys have been noted with regard to the intensity of context-

specific defensive responses. These defensive responses have been characterized using the HIP (see previous section). For example, some monkeys tend to coo frequently during the A condition (in which the animal is isolated), whereas other same-aged animals engage in little or no cooing. Large individual differences have also been observed in the duration of NEC-induced freezing (in the presence of a human profile) and ST-induced hostility (in response to direct eye contact with the human intruder). Some animals freeze the entire length of the test period, whereas at the other extreme some never freeze and act relatively undisturbed by the human intruder. These individual differences in fear-related responses seen in the laboratory are similar to those that have been observed in rhesus monkeys who inhabit Cayo Santiago, a 45-acre island with approximately 1,000 freeranging monkeys (Kalin et al., unpublished data). Importantly, it has been found that monkeys' individual differences in defensive responses are relatively stable over time, suggesting that the intensity of defensive behavior that is displayed reflects a trait rather than a state characteristic. It was initially demonstrated that the duration of NECinduced freezing behavior remained stable in 12 animals tested twice with an interval of 4 months (r = .94). Using a larger sample size, the stability of NEC-induced freezing was confirmed; ST-induced hostility was also found to be relatively stable (Kalin et al., unpublished data). Interestingly, significant correlations between the magnitude of the different types of defensive responses were not observed within an animal. Thus, monkeys that exhibited extreme levels of NEC-induced freezing did not necessarily display extreme levels of ST-induced hostility. This lack of correlation between different types of defensive responses suggests that cooing, freezing, and defensive hostility represent different and somewhat unrelated characteristics of animals' defensive styles. Pharmacologic data also support this notion. For example, manipulations of the opiate system affect A (alone condition)-induced cooing without affecting threat-induced freezing or hostility. Conversely, benzodiazepines reduce the threat-related behaviors, but have little effect on A-induced cooing (14).

Finally, to identify some of the mechanisms underlying these individual differences in defensive responding, the relationships between the stress-related hormone cortisol or asymmetric frontal EEG activity and individual differences in fearful behavior were examined. Thus, in 28 mother-infant pairs, it was found that in both mothers and infants freezing duration was significantly and positively correlated with baseline (nonstressed) cortisol levels (38). These data are consistent with findings from human studies demonstrating that extremely inhibited children have elevated levels of salivary cortisol (36,37), and is also consistent with findings in rodents that corticosterone (the rodent analogue of cortisol) is required for rat pups to develop the ability to freeze when threatened (39).

Extremely fearful monkeys (as identified by the HIP) also exhibit characteristic EEG patterns. In adult humans,

asymmetric right frontal brain activity has been associated with negative emotional responses (40). Our studies in rhesus monkeys have demonstrated similarities in this measure between monkeys and humans (41). Thus, it has been found that dispositionally fearful monkeys have extreme right frontal brain activity, paralleling the pattern of extreme right frontal activity in humans who suffer from anxiety-related disorders. In addition, it was found that individual differences in asymmetric frontal activity in nonhuman primates in the 4- to 8-Hz range are a stable characteristic of an animal (41,42). Furthermore, a significant positive correlation between relative right asymmetric frontal activity and basal cortisol levels in 50 one-year-old animals was found. As predicted, the more right frontal an animal was, the higher was its cortisol level. An extreme groups analysis revealed that extreme right compared to extreme left frontal animals had greater cortisol concentrations as well as increased defensive responses, such as freezing and hostility. The association between extreme right frontal activity and increased cortisol appeared to be long-lasting because the right frontal animals continued to demonstrate elevated cortisol levels at 3 years of age. These results are the first to link individual differences in asymmetric frontal activity with circulating levels of cortisol. This finding is important because both factors have been independently associated with fearful temperamental styles.

It has recently been found that cerebrospinal fluid (CSF) levels of corticotropin-releasing hormone (CRH), a peptide that mediates stress responses, are significantly elevated in monkeys that display exaggerated defensive responses to threatening stimuli (5). As stated before, these extreme individual differences in defensive behaviors are stable over time. Moreover, it was found that CSF CRH levels are also stable over time in rhesus monkeys. Finally, when comparing monkeys with extreme right frontal activity (that display exaggerated fearful responses) to those with extreme left frontal activity (that display low levels of fearful behaviors), the right frontal group was found to consistently have increased CSF CRH levels over a period of 4 years (5). Thus, it appears that extreme fearful behavioral responses in nonhuman primates are associated with increased levels of stress hormones such as cortisol and brain CRH, and also with extreme right frontal brain activity versus left frontal brain activity, a profile that has been found in humans suffering from stress-related psychopathology (43). Taken together, these findings suggest that in primates, a fearful endophenotype can be conceptualized as a constellation of hormonal, electrophysiologic, and behavioral characteristics. Studying species-specific defensive behaviors and their neuroendocrine and physiologic correlates offers a powerful approach for identifying animal correlates of anxiety.

Rodents

Extreme individual differences in the expression of stressrelated defensive behaviors have also been noted in rodent

species. The examination of naturally occurring genetic variations with regard to stress reactivity may have important implications for the elucidation of individual differences in sensitivity to stressful situations. One example of naturally occurring individual differences comes from the study of different rodent strains with regard to their level of stress-like behavioral responding to environmental stimuli. Because of the important role of the CRH system in regulating defensive behaviors induced by stressful or threatening situations, attention has been focused on identifying rat or mouse strains that display differential stress reactivity and different baseline levels of CRH gene expression. For example, it has been found that baseline levels of CRH messenger RNA (mRNA) are significantly higher in the amygdala of fawn-hooded rats compared to either Sprague-Dawleys or Wistars (44,45). Fawn-hooded rats have also been reported to exhibit exaggerated behavioral responses to stress such as enhanced freezing, leading to the suggestion that this strain may have utility as a model for endogenous stress-related CRH overexpression and anxiety. Strain differences, which essentially reflect differential genetic makeups, have also been found to influence the effects of acute environmental stressors on regulating CRH system gene expression. Thus, the stress of whole-body restraint produces a much larger increase in CRH mRNA levels within the hypothalamus of Fisher rats than in Wistars or Sprague-Dawleys (46,47). Similarly, the spontaneously hypertensive and borderline hypertensive strains of rats have increased basal and stressinduced levels of hypothalamic CRH mRNA compared to the Wistar and Sprague-Dawley strains (48-50).

In mice, it has been shown that the BALB/c strain is hyperresponsive to a variety of stressors compared to the C57BL/6 strain; BALB/c mice exhibit significantly higher avoidance of aversive areas in a light-dark transition test and an open field (51,52). These mice also show high levels of neophobia (53). Recent genetic mapping studies in these strains have revealed that these behavioral differences may be associated with differential levels of γ -aminobutyric acid receptor A (GABA_A) expression between the strains. For example, it has been found that BALB/c mice have significantly lower levels of benzodiazepine binding sites in the amygdala compared to C57BL/6 mice (54). As described below, alterations in the expression of GABAA receptors have been found to lead to increased anxiety-like behaviors in genetically modified mice (see CRH System Transgenic Mice).

Taken together, these findings indicate that different rodent strains, as a consequence of their distinct genetic makeups, display different baseline levels of gene expression within various systems that are known to regulate the expression of stress-induced defensive behaviors. The study of various rodent strains may thus help to identify the neurogenetic differences that contribute to individual differences in stress susceptibility, and thereby further characterize the interaction between genes and environmental conditions in the etiology of anxiety. Although such information is useful, it remains to be determined whether or not the specific genetic differences identified above actually underlie the different behavioral effects. It is probable that a number of genes in addition to those described above are differentially expressed across different rodent strains. Which other genes differ across strains, and of these, which ones contribute to the behavioral profile? It is also unclear whether the differential gene expression patterns are the cause or the result of the different phenotypes observed in the separate strains. Future studies in which behavioral phenotypes are assessed after the application of novel gene targeting techniques to selectively disrupt or restore gene function in these rodent strains will aid in clarifying these issues.

MATERNAL DEPRIVATION: AN ENVIRONMENTAL MANIPULATION THAT CAN LEAD TO FEARFUL ENDOPHENOTYPES IN PRIMATES AND RODENTS

Converging lines of evidence from a number of species point to the importance of the early postnatal period, and in particular the bond between mother and infant, in the development of normal defensive behaviors and the putative emotional states underlying these behaviors. It has been observed that children who were placed in nurseries that lacked adequate social stimulation developed a syndrome of "protest, despair, and detachment" that may be analogous to an increase in defensive responses (55). Furthermore, recent reports suggest that children reared without appropriate nurturance can display neuroendocrinologic alterations and may develop long-term behavioral and emotional difficulties including an increased risk for stress-related psychiatric illness (56,57).

Perhaps the most significant environmental factor during the early development of mammals is the interaction between the infant and its mother. As described above, separation of an infant from its mother during this early developmental phase represents a significant stressor that markedly and negatively affects the subsequent emotional development of the infant (55,57). In fact, disruption of normal attachment behavior at critical developmental phases can, in a number of species, lead to marked and persistent disturbances in behaviors and brain systems that are thought to participate in the regulation of fear-related responses; this disruption may ultimately contribute to an individual's propensity to develop exaggerated or inappropriate defensive responses.

Altered maternal-infant interactions can lead to anxiety endophenotypes in nonhuman primates and rodents, thus identifying an environmental manipulation that can be used to create animal models of increased stress-related functioning. Indeed, a large body of work in monkeys and rats indicates that a number of deleterious and long-lasting effects

are produced as a result of separating infants from their mothers prior to weaning. The notion that perturbations in the early postnatal environment might have enduring neuroendocrine, neurochemical, and behavioral effects was originally put forth several decades ago by Levine (58). It has since been demonstrated that a likely source of these alterations is a disruption of the interaction between mothers and pups (59,60).

Nonhuman Primates

The classic studies by Harlow and colleagues (20,61,62) of the effects of maternal separation in primates found that in addition to life-supporting nourishment, physical contact and comfort are necessary for primates' normal social and emotional development. During the first months of life, the attachment between mother and infant is intense, and as a consequence the infant remains in close proximity to its mother (61,63). Long-term maternal separation can result in profound alterations in stress-related behavioral responses in the separated offspring. Monkeys that have been separated from their mothers for prolonged periods during this time exhibit symptoms of enhanced defensive or fear-related behavioral responses into adulthood and appear socially withdrawn, a phenomenon that has led to the suggestion that the behavioral and neuroendocrine sequelae of maternal separation might provide a model for some of the dysfunction that is observed in anxiety disorders and depression (64-68).

Furthermore, neuroendocrine studies in rhesus monkeys indicate that an infant's stress hormone levels are negatively correlated with the number of offspring the mother had, suggesting that when mothers are less experienced, cortisol levels in their (early born) infants are high; elevated cortisol levels also correspond to increased fearful behavioral responses in the infants (38). Cortisol has been found to play an important role in mediating the development of defensive responses (69); thus, factors that were expected to affect infant primate cortisol concentrations were examined. It was found that maternal cortisol levels were moderately correlated with those of their infants (38). Interestingly, it was also found that maternal parity was negatively correlated with infant cortisol levels such that the current infants of mothers that previously had more offspring were likely to have lower cortisol levels. This finding indicates that a mother's past infant rearing and/or pregnancy experience may contribute to individual differences in infant baseline cortisol levels, and provides further support for the notion that the mother-infant interactions may be a critical factor in determining the future fearful disposition of the offspring (38). Although the precise mechanism for this interaction remains to be determined, it is likely that mothers with little rearing experience would interact differently with their infants than mothers with more experience.

Evidence for the notion that long-lasting dysregulation

of the CRH system may in part underlie the harmful consequences of early developmental stressors has been provided in a study of nonhuman primates that were exposed to adverse rearing conditions during infancy. Coplan and colleagues (70,71) found that CSF levels of CRH are basally and chronically elevated in adult bonnet macaques whose mothers were exposed for 3 months to an unpredictable variable foraging demand (VFD), in comparison to mothers confronted with either a high but predictable or low but predictable foraging demand. Infants reared by VFD-exposed mothers have been found to subsequently display abnormal affiliative social behaviors in adulthood (72). These findings are consistent with the recent results from this lab that indicate that CSF CRH levels are elevated in dispositionally fearful monkeys, and that this CRH elevation is a stable trait-like characteristic of fearful endophenotype (5).

Rats

These aforementioned findings in nonhuman primates support the notion that mother-infant interactions may be a critical factor in determining the future fearful disposition of the offspring. Maternal separation has also been found to produce long-term changes in defensive behaviors into adulthood in rats. Using the maternal separation paradigm in rats, investigators have also been able to begin to elucidate some of the alterations in gene expression that take place in response to this early life stressor.

Interestingly, the nature of the separation determines the direction of the long-term changes, as has been reviewed in detail recently (73–75). Thus, brief periods of separation (3 to 15 minutes per bout, once a day, for roughly 2 weeks) from the mother result in a profile indicative of diminished anxiety, whereas more protracted separations (3 hours or more) have the opposite effect, resulting in increased stresslike responses. In an elegant series of studies by Plotsky and Meaney (76), the long-term effects of these different types of maternal separation have been described, and the behavioral and neuroendocrine mechanisms underlying these long-term effects have been characterized. It was initially found that rat pups that underwent very short periods of separation (termed "handling") from their mothers had decreased basal levels of hypothalamic CRH mRNA and median eminence CRH immunoreactivity as adults compared to undisturbed control rats. As adults, these "handled" pups also displayed significantly lower elevations of stress-induced corticosterone levels and blunted CRH release from the median eminence relative to controls. It has since been found that the mechanism underlying this reduction in stress-related functioning in handled rat pups involves the type of maternal behavior that is displayed after the pups are returned to the mother (77), confirming earlier hypotheses that maternal behavior is the critical component in the developmental milieu of the infant (58). A brief removal of rat pups from the dam results in a significant

increase in the amount of licking, grooming, and archedback nursing (LG-ABN) that the mother lavishes on the pups when they are returned; the total amount of time spent nursing and contacting the offspring is not affected, but rather the quality of the interaction between mother and pup is altered. In nonseparated pups, individual differences in LG-ABN predict hypothalamic-pituitary-adrenal (HPA) axis responsivity in adulthood such that mothers that engage in high levels of LG-ABN have offspring that, as adults, show reduced HPA axis activation in response to stress and have decreased levels of CRH mRNA in the paraventricular nucleus (PVN) of the hypothalamus (77). Pups that are born to mothers that naturally exhibit high levels of LG-ABN grow up into adults that display low-anxiety-like behaviors (increased exploration of novel environments) and compared to low-LG-ABN offspring, have decreased levels of CRH receptors in brain regions such as the locus coeruleus that are thought to mediate stress responses (78). Taken together, these findings indicate that increased nurturing physical contact from the mother can lead to a toned-down stress-responsive system in the offspring.

In contrast, longer periods of maternal separation seem to have the opposite effect on stress-related functioning later in life. Rat pups that are separated from the mother for 3 hours or longer (investigators have often used a 24-hour separation) show in adulthood increased CRH system gene expression, exaggerated HPA axis responses to stress, and increased stress-like behaviors in paradigms such as the elevated plus maze (76,79,80). Other intense stressors such as an endotoxin insult during the perinatal stage are also able to produce marked elevations in basal CRH gene expression and lead to an exaggerated stress-induced HPA axis response in adulthood (81). It has accordingly been hypothesized that the perinatal environment plays a critical role in "programming" or "setting" the animal's stress coping system (perhaps through alterations in CRH system gene expression) for the remainder of its life (73-75). Maternally separated rats also show alterations in other systems that are known to regulate stress-related behaviors and that are consistent with an increased fearful endophenotype. For example, maternal separation increases the release of norepinephrine into the PVN of the hypothalamus in response to restraint stress; stress-induced plasma adrenocorticotropic hormone (ACTH) levels were also elevated in maternally deprived rats (82). Early life stressors such as maternal separation may therefore play an important role in determining the eventual stress-related endophenotype that is exhibited in adulthood. Moreover, the aforementioned studies provide an example of how the animal endophenotype approach can be applied to investigating molecular correlates of anxiety-related conditions.

It should be mentioned that prenatal stress can also produce alterations in indices of stress-induced responding in adulthood. For example, in rats, disturbing the prenatal environment by stressing the mother can lead to increases in

CRH gene expression in the fetal PVN, increases in CRH content in the amygdala of adult offspring, and potentiation of stress-like behavioral responses in these rats whose mothers had undergone stress during pregnancy (83–85). These findings further support the notion that mother-infant interactions may be a critical factor in determining the future fearful disposition of the offspring.

TARGETED MUTATIONS LEADING TO ANXIETY-LIKE ENDOPHENOTYPES: STUDIES OF TRANSGENIC MICE

A perhaps more direct approach for studying the genetic underpinnings of a particular animal endophenotype is to characterize the change in an organism's interaction with its environment following either overexpression or underexpression of a particular gene product. Transgenic and knockout mice are thus now widely used in the ongoing effort to understand the contributions of specific genes to psychopathology. The detailed methodology for the generation of these animals and their use in neuroscience research has been reviewed (86). Briefly, genetic alterations are introduced in the embryonic stage such that the mouse develops with the mutation, thereby putatively providing a model for congenital abnormalities that may contribute to anomalous functioning and the expression of a particular endophenotype.

Using this strategy, a variety of components within the CRH, serotonin (5-hydroxytryptamine, 5-HT), and GABA systems have been successfully targeted and studied for their roles in mediating stress-related behavioral effects (87–89). It should be noted that in addition to the aforementioned systems, there are several other important central regulators of stress and anxiety-related processes. The norepinephrine system has long been implicated in the modulation of anxiety states. Several recent reviews detail the preclinical and clinical evidence for the involvement of norepinephrine (NE) in anxiety-related disorders such as panic and posttraumatic stress disorder (90,91). Indeed, β-adrenergic receptor antagonists and α_2 -receptor agonists are effective in the treatment of certain anxiety-related symptoms in humans. In addition, recent preclinical evidence indicates that a variety of central nervous system (CNS) peptides including cholecystokinin (CCK), neuropeptide Y (NPY), and substance P/neurokinins participate in the regulation of anxiety-like behaviors (92-94).

Although these peptide and neurotransmitter systems undoubtedly play a role in stress- and anxiety-related behaviors, the focus of the following section will be the CRH, 5-HT, and GABA systems because these three systems are perhaps the most thoroughly studied with transgenic models. Although the transgenic/knockout approach has provided valuable new information about the genetic regu-

lation of stress-related fearful endophenotypes, it should be kept in mind that there are a number of important caveats regarding the interpretation of findings from transgenic animal studies (described at the end of this section). As outlined below, alterations of discrete genes within each of these systems results in an anxiety-like murine endophenotype, characterized by the increased expression of certain aspects of rodent defensive behaviors.

The CRH System

CRH and the related endogenous peptide agonist urocortin (95) bind to the two cloned CRH receptors, designated CRH1 and CRH2 (96–98), and to the CRH binding protein (CRH-BP) (99). The CRH-BP has been postulated to function as an endogenous buffer for the actions of the CRH family of ligands at their receptors (100). CRH, its receptors, and its binding-protein are expressed in key structures of the HPA axis, and thereby participate in mounting the neuroendocrine response to environmental perturbations. The various elements of the CRH system are widely and heterogeneously expressed in cortical, limbic, and brainstem structures and these regions are thought to regulate behavioral responses to stress.

CRH System Transgenic Mice

Given that central infusion of CRH results in enhanced fear-related defensive behaviors, CRH-overexpressing mice were predicted to display increased stress-like behavioral responses. Indeed, these mice have been found to have several behavioral effects associated with acute CRH administration. CRH overexpressing mice exhibit a profile that is consistent with increased levels of stress, such as reduced baseline and stress-induced exploration of a novel environment, and decreased activity and time spent in the open arms of an elevated plus maze (101,102). These effects are potently blocked by administration of the CRH receptor antagonist α-helical CRH. CRH transgenic mice also show a profound decrease in sexual behaviors and significant deficits in learning; higher order functions such as these are typically abolished when a situation is found threatening and defensive behaviors are recruited (103,104). Thus, CRH overexpressing transgenic mice may represent a genetically engineered model of a murine anxiety-like endophenotype.

Consistent with the notion that heightened CRH transmission elicits stress-like behaviors is the finding that CRH-BP knockout mice show increased stress-like behaviors. CRH-BP knockout mice displayed decreases in open arm entries and open arm time in an elevated plus maze, and showed a decrease in the number of exits from a safe box in a defensive withdrawal/open field paradigm (105). These results indicate a heightened level of neophobia in these mice. Moreover, CRH-BP knockouts have reduced body weight gain over several weeks (105,106), which is also syn-

tonic with increased basal CRH activity. The behavioral profile of CRH-BP knockouts is similar to that which is seen with exogenous CRH administration (107), and supports the notion that removal of the CRH-BP may lead to increased basal stress responses due to increased CRH tone. Thus, as with the CRH-overexpressing mice, CRH-BP knockouts may represent another rodent anxiety-like endophenotype. In terms of the predictive validity of these models, CRH receptor antagonists have been found to block the stress-like behavioral profile observed in these animals; one recent clinical study indicates that CRH1 receptor antagonists may indeed prove to be effective anxiolytics or antidepressants (108).

Interestingly, deletion of the CRH gene does not appear to decrease stress-like behaviors, as might be predicted from the aforementioned work. Although certain endocrinologic deficits are observed in CRH knockout mice, stress-related behavioral function in these animals remains relatively unaffected as assessed by multiple stress-related paradigms (109-114). This sparing of normal stress responsivity may be due to compensatory increases in the expression of other CRH system ligands such as urocortin. Deletion of the CRH1 receptor gene, however, does appear to consistently result in a putative reduction in anxiety (115-117). For example, CRH1 knockout mice show increased exploration of the open arms on an elevated plus maze and spend more time in the brightly lit compartment of a dark-light transition box than do wild-type controls. Moreover, CRH1 knockout mice appear to be immune to the anxiogenic effects of ethanol withdrawal (117). Studies of CRH2 receptor knockout mice, on the other hand, indicate that these mice display a less consistent behavioral profile than the CRH1 knockout mice (118-120). Part of the behavioral profile of CRH2 knockouts is suggestive of increased stresslike responding, but other aspects of the behavioral profile indicate either no alteration of stress-related responding (118,119), or a decrease in anxiety-like behaviors (120). The observed increases in anxiety-like behaviors in these genetically altered mice may be due to increased levels of brain CRH and/or urocortin; in two of the three studies, an elevation of baseline CRH or urocortin mRNA levels in the CNS was seen in CRH2 knockout mice (118,119). Thus, the endophenotype displayed by CRH2 knockout mice may actually be indirectly due to a compensatory alteration induced by the mutation rather than simply due to a lack of CRH2 receptor expression. It should be noted that acute blockade of CRH2 receptors results in a decrease in stress-induced defensive behaviors; thus the behavioral profile of these animals is opposite to that of mice that are missing the CRH2 receptor (121,122). Thus, the timing of the gene deletion may critically influence the nature of the behavioral phenotype that ensues. Future studies utilizing novel inducible-knockout technologies may help in clarifying the developmental versus acute role of various genes in the development of anxiety-related endophenotypes (123).

Clinically Effective CRH System Drugs for Stress-Related Disorders

A large body of preclinical literature indicates that CRH is a critical modulator of stress and anxiety-like behaviors in nonhuman primates and rodents (107,124). Based on the ability of CRH1 receptor-selective antagonists to block many of the behavioral effects of stress or CRH administration, these antagonists have been proposed as potentially therapeutic agents for the treatment of stress-related psychiatric conditions including anxiety and depression (125). Pharmacologic analysis of stress-induced primate defensive responses has also revealed that the CRH system is a critical modulator of this index of anxiety-related behavior. For example, administration of CRH into the cerebral ventricles of nonhuman primates results in a constellation of behavioral responses that closely resemble the defensive responses that are exhibited upon presentation of a stressor (124). Consistent with the notion that increased levels of CRH are associated with increased anxiety-like responding are the recent findings that small-molecule CRH1 receptor antagonists block the expression of some behavioral, physiologic, and neuroendocrine responses to stressors in rhesus monkeys (126,127). The first report of an open-label clinical trial with a CRH1 antagonist was recently published, and revealed a significant effect of this compound in ameliorating symptoms of depression and anxiety (108). Although further research is needed to firmly establish the utility of CRH1 antagonists as psychotherapeutic agents and also to determine the possible side effects associated with their use, these preliminary data support the notion that these compounds represent an important new class of drugs that may offer great promise for the treatment of illnesses associated with increased anxiety and stress.

The 5-HT System

Serotonin is a member of the monoamine family of transmitters that also include dopamine and norepinephrine. As is typical for the monoamines, cell bodies for this neurotransmitter are found in discrete nuclei within the midbrain (dorsal and medial raphe nuclei) and send widespread 5-HT–containing projections throughout the brain (128). 5-HT produces its effects through at least 15 different 5-HT receptors that are differentially distributed throughout the CNS; the principal mode of 5-HT inactivation is cellular reuptake via terminal transporter proteins (129). The 5-HT system has long been implicated in the regulation of mood states and anxiety, and selective serotonin reuptake inhibitors (SSRIs) constitute a major class of antidepressants that have anxiolytic effects. As outlined below, 5-HT transmission also plays a critical role in the regulation of anxiety-

like behaviors. Given the plethora of 5-HT receptors and the paucity of highly selective ligands for these multiple target sites, several investigators have employed murine gene targeting strategies to elucidate the roles of specific 5-HT receptors in the regulation of stress and anxiety.

5-HT System Transgenic Mice

Studies of targeted gene deletions within the 5-HT system have revealed an important role for this system in the regulation of stress and anxiety-related behaviors in mice. The behavioral sequelae of disrupting 5-HT receptor gene expression have been elegantly summarized in several review articles (89,130–132). Perhaps the best-characterized 5-HT mutant mice are the 5-HT_{1A} and 5-HT_{1B} receptor knockouts. Mice with a mutation in the 5-HT_{1A} receptor gene have been found to display increased stress-like behaviors in multiple tests of approach-avoidance conflicts. These animals show decreased entries into and time spent in the more aversive region in paradigms such as the open field, elevated plus maze, and the elevated zero maze; thus 5-HT_{1A} knockout mice avoid the center of an open field, the open arms of a plus maze, and the unenclosed regions of a zero maze (133–135). It is worth noting that this "increased anxiety" pattern of results was found consistently across three different research labs, indicating its robustness and reproducibility. Consistent with this profile is the finding that these mice also exhibit decreased activity in the presence of and approach toward a novel object (135). This increase in stress-like responding is not accompanied by changes in overall locomotor activity or motor and spatial coordination, as assessed in photocell cages and a RotaRod apparatus. Curiously, 5-HT_{1A} knockout mice display increased mobility in response to an acute stressor such as forced swimming or tail suspension (133-135). Taken together, these findings indicate that 5-HT_{1A} knockout mice may represent another animal endophenotype of increased anxiety.

The constitutive knockout of the 5-HT_{1A} receptor does not seem to lead to compensatory alterations in the expression of serotonin or its transporter, or to changes in catecholamine levels in several brain regions (135). Interestingly, a recent report indicates that this mutation alters GABA system expression and function (136). It has been found that anxiety-like behaviors in 5-HT_{1A} knockout mice are relatively unaffected by benzodiazepine treatment. Analysis of brain tissue from these animals indicates that GABAA receptor binding is reduced and that the expression of α_1 and α_2 subunits of the GABA_A receptor are decreased in the amygdala. The anxiolytic actions of benzodiazepines may in part be mediated by GABA_A receptors within the amygdala; the profile of results in 5-HT_{1A} knockout mice has led to the intriguing speculation that the anxiety-like endophenotype in these mice may actually in part derive from a decrease in the expression and function of the GABAA receptor (136). This proposed mechanism is consistent with the increase in stress-related behaviors that are seen in certain transgenic mice with mutations in the GABAA receptor (see below). Further work is necessary to determine the precise mechanisms through which the developmental interruption of 5-HT_{1A} gene expression results in the observed anxiety-like endophenotype.

In contrast to 5-HT_{1A} knockout mice, mice that lack the 5-HT_{1B} receptor show decreased anxiety-like behaviors in several tests of approach-avoidance conflicts. 5-HT_{1B} knockout mice spend more time in the center of an open field and more readily explore novel objects than their wildtype controls; this profile is opposite from that of 5-HT_{1A} knockout mice, and is suggestive of diminished neophobia (89,137). Consistent with this pattern of results is the finding that as pups, 5-HT_{1B} mice emit fewer ultrasonic vocalizations when separated from their mothers; separationinduced vocalizations are thought to provide a measure of anxiety and distress in pups (138,139). It is interesting to note, however, that no changes in contextual or cue-induced conditioned freezing are observed in 5-HT_{1B} mutant mice, suggesting that approach-avoidance conflicts and conditioned fear may be differentially modulated by the 5-HT system. The other main behavioral effect of constitutive 5-HT_{1B} receptor deletion is a marked increase in aggressive behavior (89,140,141). Given that aggressive behaviors represent an important part of an organism's response to threat, 5-HT_{1B} knockout mice may also provide valuable information on the neural and genetic factors associated with stress and anxiety-related functioning (89,142).

It should be noted that mice with null mutations of other 5-HT receptor subtypes have also been generated, but these animals have not been found to display as robust an anxietyrelated behavioral profile as the 5-HT_{1A} or 5-HT_{1B} knockout mice. It has been found that 5-HT_{5A} receptor knockout mice show increased exploratory activity in the presence of novelty, but do not differ from wild-type controls with regard to avoidance behaviors from an aversive environment such as the open arms of a plus maze, or the center of an open field (143). These knockout mice also do not respond differently from control subjects in tests of startle reactivity or in burying a probe that delivered a brief electric shock. Thus, these animals appear to have yet a different behavioral profile from that of the 5-HT_{1A} or 5-HT_{1B} knockout mice. An initial report indicates that 5-HT₆ receptor deficient mice may exhibit increased avoidance of aversive environments; although these preliminary findings are interesting, further work is needed to fully characterize the phenotype of these mutant mice (144,145). Mice lacking either the 5-HT_{2A} or 5-HT_{2C} receptors have also been created; to the best of our knowledge, the stress-related behavioral functioning of these animals has yet to be reported (146,147).

Clinically Effective 5-HT System Drugs for Stress-Related Disorders

As mentioned above, one of the most commonly prescribed and effective classes of drugs that is used in the treatment of depression and anxiety is the SSRIs, which block the reuptake of 5-HT by its transporter and thereby increase serotoninergic transmission. Based on the findings of preclinical studies including those obtained from 5-HT receptor knockout mice, 5-HT_{1A} agonists have been developed for the treatment of anxiety. The clinical utility of this class of compounds, however, remains to be determined. As these transgenic approaches develop and become more refined, they will undoubtedly aid in clarifying the roles of the many other 5-HT receptor subtypes in processes related to stress and anxiety and will aid in drug development.

The GABA System

The primary inhibitory neurotransmitter in the CNS is GABA; GABA-synthesizing cells are distributed throughout the brain (128). The actions of GABA are mediated by two major classes of receptors, GABA_A and GABA_B, both of which modulate the activity of ion channels. The principal mode of inactivation of GABA transmission is the presynaptic reuptake of GABA by its transporter protein. Although both types of GABA receptors are widely distributed through the CNS, several important differences exist between the two. Relevant to psychopharmacology is the finding that traditional anxiolytics (benzodiazepines) do not bind to GABA_B receptors, but rather mediate their effects through GABAA receptors. GABAA receptors consist of a chloride channel formed by the pentameric arrangement of at least 18 different protein subunits (α_{1-6} , β_{1-4} , γ_{1-3} , δ , ϵ , π , ρ_{1-3}), thus allowing for considerable heterogeneity of the GABA_A receptor isoforms (148). Typically, benzodiazepine-responsive GABA_A receptors consist of α , β , and γ subunits; in addition to the benzodiazepine site, these receptors also contain distinct sites for the binding of GABA, barbiturates, and ethanol. These various regions act as allosteric regulators of GABA-induced chloride channel opening. Although psychotherapeutic effects such as anxiolysis are achieved through facilitation of GABA transmission at this receptor, drugs that act as GABAA receptor agonists also produce several deleterious side effects. The extent to which differences in GABA_A receptor subunit composition might contribute to possible dissociations between the beneficial and negative effects of these compounds is currently being investigated.

GABA System Transgenic Mice

The synthesis of GABA is regulated by two isoforms of the enzyme glutamate decarboxylase (GAD), GAD67, and the

shorter form GAD65 (149). Whereas GAD67 is thought to maintain basal GABA levels, GAD65 is thought to regulate the synthesis of GABA at nerve terminals in response to high GABA demand (150). Given the important role of GABA in inhibitory neurotransmission associated with anxiolysis, several investigators have evaluated the behavioral profile of genetically altered mice that lack the GAD65 gene. Two separate groups have reported that GAD65^{-/-} mice display an increase in stress-like behaviors in numerous behavioral paradigms (151,152). GAD65 knockout mice had fewer entries into and time spent in the center of an open field or the open areas of an elevated zero maze (similar to an elevated plus maze), indicating that they were more avoidant of inherently aversive areas. Similarly, these mice had lower levels of activity in the bright portion of a lightdark transition box. It should be mentioned that GAD65^{-/-} mice also displayed an elevation in the occurrence of spontaneous and stress-induced seizures, and that these mice had a dramatically increased mortality rate starting at 4 to 5 weeks after birth (151). Thus, although the behavioral profile of GAD65 knockout mice is suggestive of increased anxiety-like responses, it is possible that these effects are secondary to the occurrence of seizures and to the factors leading to early lethality. The usefulness of this knockout as a model for anxiety-related deficits may therefore be limited. Given that benzodiazepines and barbiturates act as positive modulators of GABA transmission at the GABA_A receptor by enhancing GABA-induced chloride channel opening, it is of interest to note that GAD65^{-/-} mice were not sensitive to the effects of either benzodiazepines or barbiturates, but did respond to the direct GABA_A agonist muscimol, which binds directly to the GABA site of the GABA_A receptor and increases opening of the chloride channel in the absence of GABA (152). This pharmacologic profile is consistent with the finding that GABA synthesis is blocked by the GAD65 null mutation, but that GABAA receptor binding is unaffected by this change. Furthermore, this mutation does not seem to alter the functioning of GABA receptors because direct agonists stimulate the receptor but indirect modulators of GABA do not.

In an attempt to delineate the roles of the various GABA_A receptor subunits in the regulation of stress- and anxiety-related behaviors, investigators have generated mutant mice with alterations in the expression of specific GABA_A receptor subunits. It was initially reported that deletion of the γ_2 subunit led to a selective (94%) reduction in the expression of benzodiazepine sites in the CNS without alterations in the level of GABA sites or changes in the expression of other GABA_A receptor subunits (153). Thus, γ_2 knockout mice possessed functional GABA_A receptors that responded normally to GABA site ligands or barbiturates, but did not respond to benzodiazepines; these findings led to the conclusion that the γ_2 subunit is not necessary for the formation of functional GABA_A receptors, but is required to create

the benzodiazepine-responsive site of those receptors. Mice that were homozygous for the mutation, however, did not live past weaning in this study. In mice carrying only one copy of the functional γ_2 gene, a 20% reduction in benzodiazepine sites was observed, but these mice did not show overt developmental deficits. In a recent study, a detailed characterization of the behavioral profile of these animals was carried out. Heterozygotes displayed a decrease in the number of entries into and amount of time spent in the open arms of an elevated plus maze and the bright compartment of a light-dark box. These animals also exhibited a decrease in the exploration of novel areas, and an increase in certain forms of fear conditioning that are thought to be mediated by the hippocampus. Finally, γ_2 heterozygotes were found to react to partially conditioned stimuli (only weakly paired with aversive consequences) as if they were full and potent predictors of threat; compared to wild-types, which showed low levels of defensive behaviors to the partially conditioned stimulus, heterozygotes displayed high levels of conditioned freezing to the partial conditioned stimulus that were identical to those displayed by all animals in response to the full conditioned stimulus. This profile has been proposed to be a model for the tendency to interpret neutral situations as threatening that is seen in anxiety patients. Taken together, the results from this extensive behavioral profile indicate that $\gamma_2^{+/-}$ mice have increased neophobia and stress-like responses and may thus provide a model for increased anxiety-like behaviors (154-156). Interestingly, all of the elevations in stress-like behaviors in γ_2 heterozygotes were blocked by the benzodiazepine diazepam, suggesting that this animal model may also have good predictive validity for identifying clinically effective anxiolytics.

It is also extremely important to mention the α_1 subunit transgenic mice, whose behavioral profiles have been thoroughly and insightfully reviewed in recent articles (157, 158). In these mice, a single amino acid is altered (histidine replaced by arginine at the 101 position of the peptide) in the α_1 subunit of the GABA_A receptor complex. This subtle change does not produce any overt alterations in baseline responses to stress in the genetically altered mice; these animals behave similarly to wild-type controls in tests such as the elevated plus maze and the fear-potentiated startle paradigm, a measure of conditioned fear (159,160). Thus, under drug-free, normal conditions, these animals do not display a behavioral pattern that is consistent with an anxiety-like endophenotype. When these mice are treated with conventional benzodiazepines, however, they react very differently to the drug than their wild-type counterparts. Mice with the mutation in the α_1 subunit display a normal reduction of stress-induced anxiety-like behaviors after benzodiazepine treatment, but fail to display some of the more deleterious side effects associated with this class of drugs such as sedation, amnesia, and ataxia. These results indicate that the anxiolytic effects of benzodiazepines can be separated from the negative side effects of these compounds, and that the α_1 subunit of the GABA_A receptor is likely to mediate some of these potentially harmful properties of benzodiazepines. Interestingly, McKernan and colleagues (160) demonstrate that a novel benzodiazepine-site ligand that binds to GABA_A receptors containing α_2 , α_3 , or α_5 subunits but avoids receptors with the α_1 subunit produces a behavioral profile that is identical to that of the α_1 subunit knockout mice; in normal mice, this compound decreases murine anxiety-like behaviors without eliciting sedation or ataxia (160).

Clinically Effective GABA System Drugs for Stress-Related Disorders

As stated above, the most widely used GABA system-based drugs for the treatment of anxiety are the benzodiazepines, which facilitate GABA transmission through the GABAA receptor. As outlined in the previous section, the search for novel compounds that may act selectively at specific GABAA subunits is ongoing, with the ultimate hope of discovering ligands that produce anxiolysis but do not cause some of the serious side effects that are commonly associated with benzodiazepines. As demonstrated by McKernan and colleagues (160), drugs that selectively target certain GABAA receptor subunits may hold great promise for the treatment of anxiety without harmful side effects. This development would represent a major breakthrough in the pharmacotherapy of anxiety-related disorders. The use of targeted genetic alterations in identifying the roles of various GABAA subunits will undoubtedly aid in this effort to create "designer drugs" for the treatment of anxiety (158).

General Issues and Caveats of Transgenic Animal Studies

As mentioned above, mice carrying certain mutations within either the CRH, the 5-HT, or the GABA system display an anxiety-like endophenotype. It appears that these genetically engineered mouse models also have some predictive validity; the stress-like endophenotype observed in at least two of the aforementioned models is normalized by administration of a clinically effective antianxiety agent that acts within the system that was genetically targeted. It remains to be determined, however, the extent to which these genetically altered models serve to identify potential antianxiety agents from different chemical classes. For example, do benzodiazepines reduce stress-like effects of CRH overexpressers? The extent to which the stress-like endophenotype in these animals is altered by compounds that act on systems that were not directly targeted by the genetic mutation will aid in determining the generalizability and utility of these models as predictors of novel anxiolytic agents. If one assumes that these animals provide a model of inherent trait-like anxiety, they can serve as a powerful tool for

screening new potential anxiolytics. These models do provide a sound approach to study the long-term effects of congenital abnormalities in these neurotransmitter and neuropeptide systems.

Several broad issues should be considered when interpreting studies utilizing genetically altered mice. Generally, the hypotheses regarding the behavioral profiles of transgenic mice are based on earlier findings from psychopharmacologic studies. For example, within the CRH field, the prediction that CRH overexpressers would display increased anxiety-like behaviors was based on the observation that CRH administration produces stress-like behaviors in rodents and primates (107,124). When the outcome of the transgenic studies agrees with the psychopharmacologybased prediction, the findings are taken as a confirmation of that hypothesized mechanism of action. When the outcome of the transgenic studies disagrees with the predicted phenotype, however, concerns about possible developmental confounds are raised. One of the most commonly cited drawbacks of the transgenic/knockout strategy is that the gene of interest is altered from the embryonic stage, therefore possibly influencing other genes involved in the normal development of the animal. Thus, it is difficult to tease apart the effects of under- or overexpression of that gene on the endpoints under study from effects due to compensatory or downstream developmental changes that may have occurred as a result of the mutation (86,87,161). Therefore, the transgenic/knockout approach provides an excellent method for modeling a congenital abnormality that leads to a disease state, but this approach may be less useful for identifying the discrete functions of a specific gene product because of the problems of interpretation that arise from the developmental confound. Indeed, with regard to all of the studies discussed in this section on genetically altered mice, it will be important in future studies to delineate the compensatory alterations that occur in response to the congenital mutation, and that may indirectly contribute to the adult endophenotypes that are reported for these animals. Future studies utilizing novel inducible-knockout strategies will circumvent the developmental issue; inducible knockouts may thus become a valuable tool for exploring the functions of discrete gene products for which no selective ligands are available (123).

It should also be noted that there is a large literature concerning the use of antisense oligonucleotide infusions to knock down the expression of particular gene products that may be related to fearful endophenotypes. The antisense oligonucleotide approach, however, has been plagued with a number of issues regarding toxicity, and may therefore not represent the optimal method for studying gene function *in vivo* (162).

FUTURE DIRECTIONS

Although the studies summarized in this chapter have contributed a great deal of knowledge about some of the genetic

contributions to the development of stress and anxiety-like endophenotypes in animals, further information is needed to understand the precise nature of gene-environment interactions in stress regulation. It is likely that a particular stressor results in alterations of gene expression in myriad systems and that the overall response to stress involves the coordination of gene activation and/or suppression within these various systems. Novel high-throughput technologies have recently been developed that enable the expression of thousands of genes to be assayed at once. "Gene chips" and "DNA arrays" are two powerful new tools for analyzing complex multilocus genetic interactions associated with a particular environmental perturbation or disease state (163, 164). This approach and its application to psychiatry research have been discussed comprehensively in a recent review article (165). Briefly, gene chip and DNA array technology involve the hybridization of gene transcripts from a tissue sample onto a glass slide or filter that contains up to 10,000 different nucleotide sequences. The amount and pattern of the signal hybridized to the array are then assessed; this method thus permits a rapid analysis of changes in the expression of multiple genes. This technology can also be used to identify single nucleotide polymorphisms in a particular gene by comparing the hybridization patterns of samples from different candidate populations on chips that contain multiple copies of the gene of interest, each copy differing from the previous one by just one base in the sequence. Theoretically, depending on the size of the gene, it would be possible to carry out a base-by-base examination of the entire gene on a single gene chip. However, it is important to realize that although a broad approach can be taken with this technology, it may not be sensitive enough to detect small but functionally important changes in gene expression. This technology can be applied to preclinial and clinical questions regarding the complex genetic control of stress and anxiety by examining event-related gene expression changes and also baseline differences in gene sequences (polymorphisms) that might contribute to differential stress responsivity (165). This technique, along with the recent completion of the Human Genome Project, not only raises the potential to simultaneously profile multiple gene expression systems at once, but also holds great promise for the identification of completely novel genes in stress regulation and anxiety.

A greater challenge, however, is the elucidation of the functional role of these new genes in processes related to stress and anxiety. Given this daunting task, methods for more specific and long-term gene targeting will increasingly gain importance in neuroscience research aimed at uncovering genetic dysregulation relating to psychopathology. One technique that is likely to be helpful is that of virally mediated gene transfer. In this method, a gene of interest is cloned into viral vector (with most of the viral genome removed to reduce toxicity and infection) and the modified vector is then infused into a particular brain region using

standard stereotaxic procedures (see ref. 166 for review). Depending on the gene insertion and the selection of the promoter to drive the expression of the gene, it is possible to obtain either an increase or decrease in the amount of protein resulting from the gene of interest. This method allows for highly selective gene regulation and thus provides a valuable new tool with which to study the effects of a particular gene product on stress-related functioning. The virally mediated gene transfer approach also has certain advantages over the current transgenic and antisense oligonucleotide strategies: it can be administered to the animal at any time or into any brain region, it results in a fairly robust and long-lasting up- or down-regulation of the gene, and it can be used to insert several genes at once in the same animal. Thus, the viral gene transfer approach completely avoids the issue of developmental confounds, which are perhaps the most commonly cited problems that plague current transgenic and knockout approaches. A few groups have already reported successful long-term up-regulation or down-regulation of discrete gene products related to neuroscience research applications; the behavioral effects associated with this technique appear to be quite robust and do not appear to be associated with the high level of toxicity that has been reported with antisense oligonucleotides (167–169). Thus, these methods may provide valuable new strategies to more rapidly uncover the neurogenetic basis for stress-related psychopathology.

On the clinical side, human genomic studies are indicating the existence of polymorphisms in the regulatory region of the gene encoding CRH (170-172). As careful analysis of genes for the other elements of the CRH system progresses, it will be interesting to see if particular mutations can be associated with stress-related disease states. This method has been applied successfully to study the role of the serotonin (5-HT) system in anxiety disorders; reports of polymorphisms in the gene encoding the 5-HT transporter have been made in patients with anxiety-related traits (173-176). Clinically, one challenge will be to develop more discrete definitions of anxiety-related dysfunction that will optimize the screening of patient populations for abnormalities in genes that are believed to be related to stress and anxiety (177). Moreover, gene chip technology applied to animal analogues of stress endophenotypes may provide a rapid and comprehensive method for identifying novel gene candidates for stress-related disorders. Using these methods, it may be possible in the near future to have even greater crosstalk between animal studies and clinical findings. These combined efforts will undoubtedly facilitate our understanding of the interactions between environmental and genetic contributions to anxiety and stress-related disorders.

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