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Molecular Pathways of Anxiety Revealed by Knockout Mice

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Abstract

Anxiety is a normal reaction to threatening situations, and serves a physiological protective function. Pathological anxiety is characterized by a bias to interpret ambiguous situations as threatening, by avoidance of situations that are perceived to be harmful, and/or by exaggerated reactions to threat. Although much evidence indicates the involvement of the γ-aminobutyric acid, serotonin, norepinephrine, dopamine, and neuropeptide transmitter systems in the pathophysiology of anxiety, little is known about how anxiety develops and what genetic/environmental factors underlie susceptibility to anxiety. Recently, inactivation of several genes, associated with either chemical communication between neurons or signaling within neurons, has been shown to give rise to anxiety-related behavior in knockout mice. Apart from confirming the involvement of serotonin, γ-aminobutyric acid, and corticotrophin-releasing hormone as major mediators of anxiety and stress related behaviors, two novel groups of anxiety-relevant molecules have been revealed. The first group consists of neurotrophic-type molecules, such as interferon γ, neural cell adhesion molecule, and midkine, which play important roles in neuronal development and cell-to-cell communication. The second group comprises regulators of intracellular signaling and gene expression, which emphasizes the importance of gene regulation in anxiety-related behaviors. Defects in these molecules are likely to contribute to the abnormal development and/or function of neuronal networks, which leads to the manifestation of anxiety disorders.

Abbreviations: 5-HT, 5-hydroxytryptamine; 5HTR, 5HT receptor; $A_{2a}R$, adenosine 2a receptor; ApoE, apolipoprotein E; CaMKII, α-calcium/calmodulin-dependent protein kinase II; CCK, cholecystokinin; COMT, catechol-O-methyl transferase; CRE, cAMP-responsive element; CREM, CRE modulator protein; CRH, corticotrophin-releasing hormone; CRHR, corticotrophin-releasing hormone receptor; DA, dopamine; EPM, elevated plus maze; FMRP, FragileX mental retardation protein; GABA, γ-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; GAD, glutamic acid decarboxylase; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal system; NCAM, neural cell adhesion molecule; NE, norepinephrine; nAChR, nicotinic acetylcholine receptor; NPY, neuropeptide Y; OFQ/N, orphanin FQ/nociceptin; PKC, protein kinase C.

Index Entries: Anxiety; anxiety behavior; knockout mice; serotonin; GABA; corticotrophin-releasing hormone.

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Anxiety in Humans and Animals

Concept of Anxiety in Humans and Animals

Anxiety is the subjective feeling of heightened tension and diffuse uneasiness. It is a normal reaction to threatening situations, and serves a physiological protective function in eliciting avoidance behaviors in both humans and animals. However, anxiety can be considered as pathological when an individual displays an exaggerated response to a perceived threat, or when there is a bias to interpret ambiguous situations as threatening.

Anxiety is common, affecting 5–10% of the population, and encompasses several different disorders, which are characterized by a diverse range of symptoms that are triggered in a variety of situations. Major types of human anxiety disorders include panic disorder, social phobia, obsessive-compulsive disorder, posttraumatic stress disorder, and generalized anxiety disorder. The major differences between these disorders are outlined below, and more-detailed accounts of symptoms and diagnostic criteria can be found elsewhere *(1,2).* Individuals with panic disorder experience unexpected panic attacks (chest pain, shortness of breath and palpitation), with associated cognitive symptoms (fear of heart attack or stroke). Patients suffering from social phobia are fearful and often avoidant of social situations (eating and/or speaking in public) or, more generally, to all situations involving interpersonal contact. Obsessive-compulsive disorder is characterized by repeated intrusive, unwanted, often senseless or repulsive thoughts. The obsession often takes the form of contamination (by germs or dirt), and is accompanied by rituals intended to reverse or neutralize the obsessional fear (e.g., washing). Posttraumatic stress disorder occurs in individuals after traumatic events, such as military combat, rape, assault, and serious accident. Patients experience distressing recollection of the event, and persistent arousal, particularly in response to cues reminding them of the event. Finally, generalized anxiety disorder is characterized by persistent (over 6 mo) excessive worry, inability to control worry, muscle tension, irritability, and sleep disturbance, which is not necessarily related to a specific threatening situation.

In an attempt to understand the mechanisms underlying human anxiety, many animal behavioral models have been devised *(3–5).* Generally, a fearful situation is used to elicit an avoidance or anxiety-like behavior in rodents. The tests most often used to identify an anxiety-like phenotype in mice with gene mutations are described below. The elevated plus-maze (EPM) *(6)* consists of a cross with opposing pairs of arms, which are either open or enclosed. The maze is raised off the ground, and evenly illuminated. The normal behavior of the animal is to stay in the enclosed compartment of the maze, which is less fearful. However, during normal exploratory activity, the animal will enter the open arms. Usually, the number of entries into, and the time spent in, the open arm are counted, and used to assess the anxiety level, although additional, more complex behaviors can also be recorded *(7).* Anxiety can also be assessed in the center of a brightly lit open field *(8).* Animals tend to stay and move around the periphery of the field, since the open area and bright light are aversive. The anxiety measured in an open field is assessed by the time spent in the center of the open field or the path length in this area, compared to the rest of the open field. An adaptation of this test is the light–dark crossing task *(9),* which consists of a two-compartment box, in which one area is dark, and the other is brightly lit. This test uses the animals' natural tendency to prefer the dark, and to avoid the brightly lit area. In this case, the number of crosses into, and the time spent in, the light compartment reflects the level of anxiety.

Anxiety can also be measured in conditioned-fear paradigms *(10),* such as fear-potentiated startle and contextual-fear conditioning, which involve an element of emotional learning. In these tests, a neutral stimulus, such as a sound or a light, is paired with an electric foot shock. After a few trials, the previously neutral

stimulus becomes aversive when presented alone. In animals in which learning behavior is intact, mobility and freezing time can be used as indices of anxiety. The EPM, open field, and light–dark box tests are viewed as straightforward and relatively simple tests to conduct, and as such are frequently used in the analysis of knockout mice. However, more complex methods, which highlight different aspects of anxiety-related behaviors, are available, but are rarely used in this field. For example, in the social interaction test, detailed studies of normal behavior, such as sniffing, grooming, mounting, and contact, are monitored and used to infer changes in anxiety *(11).*

How can animal models of anxiety help in gaining insight into the pathogenesis and pathomechanism of anxiety? A major caveat of animal models of anxiety is that they are based on the rodents' normal fear reaction, but human anxiety is the reflection of a pathological response to a real or perceived stimulus. Using rodent strains whose response to anxiogenic stimuli is exaggerated, like anxiety disorder patients, would be more appropriate in anxiety-related studies. Indeed, there has been an effort to select and breed individuals from rodent colonies with high anxiety-like behavior. The Maudsley reactive inbred rat strain shows a stable and reproducible deficit in exploratory behavior, compared to the Maudsley nonreactive strain *(12).* Similar differences in anxiety-like behavior have been described between recombinant inbred mouse strains *(13).* These differences in behavior arose fortuitously, and presumably reflect contributions from multiple genetic loci. Since genetic predisposition plays a role in the development of anxiety, these rodent lines can also be considered to be ethologically relevant anxiety models. However, identifying specific genes involved in anxiety behaviors in these inbred rodent strains is a complex and difficult process.

Recently, it has become possible to target specific genes that have been implicated in anxiety. Studies of patients with anxiety disorders have shown that multiple brain regions and neurotransmitter systems are likely to be involved in the pathogenesis of anxiety. Indeed, many of the genes selected for targeted mutation in mice were components of neurotransmitter systems. In addition, anxiety is a relatively common behavioral phenotype in knockout mice, suggesting that previously unidentified pathways could contribute to an increased susceptibility to anxiety.

Neuroanatomy of Anxiety

Anxiety is a complex emotion involving heightened arousal, muscle tension, and association with memories of fearful events, which involves a complex interaction between many interconnected brain regions, with each component playing a specific role *(14).* The principal components are outlined here. Brainstem nuclei are important in the regulation of levels of arousal. Of particular importance in anxiety are the noradrenergic locus coeruleus and the serotonergic raphe nuclei *(15),* but it is the amygdala that is of central importance in acquisition, retention, and expression of conditioned fear *(16–18).* The amygdala seems to function as an emotional–cognitive interface, receiving sensory information via projections from the cortex and the thalamus. Outputs from the amygdala to the frontal cortex are related to the conscious perception of fear; outputs to the locus coeruleus, hypothalamus, periaquaductal grey, and striatum mediate autonomic, neuroendocrine, and skeletal-motor responses associated with fear and anxiety. The amygdala also has connections with the septohippocampal system, which has been identified as being essential for the sensory processing of stimuli based on novelty and punishment, indicating that it could play an important role in anxiety disorders *(19).*

Neurotransmitter Systems Involved in Anxiety

Neurotransmitter systems involved in anxiety include γ-aminobutyric acid (GABA), serotonin (5-hydroxytryptamine [5-HT]), nor-

epinephrine (NE), dopamine (DA), and neuropeptides, such as corticotrophin-releasing factor or hormone (CRH), cholecystokinin (CCK), and neuropeptide Y (NPY). Many of these have been identified as sites of action for drugs that have proved to be effective in treating anxiety disorders.

Alterations in $GABA_A$ receptor ($GABA_AR$) function have been implicated in anxiety disorders. In patients suffering from panic disorder and generalized anxiety disorder, a deficit in $GABA_ARs$ has been identified in the hippocampus and parahippocampus in position emission tomography studies *(20–22).* Furthermore, $GABA_AR$ antagonists can elicit anxiety in patients with panic disorder, thereby mimicking a functional deficit of $GABA_ARs$ *(23).* Similar pharmacological manipulations of the GABA system in animals results in anxiety-like behavior *(24–26).* More recent work suggests that alterations in specific subunits of the $GABA_AR$ are associated with certain forms of anxiety, such as withdrawal-induced anxiety *(27,28).* In addition, it has long been known that benzodiazepines, which facilitate the action of GABA at the $GABA_ARs$, are very effective in a number of anxiety disorders. By using mouse lines to introduce point mutations in the α-subunits of $GABA_ARs$, the pharmacological profile of benzodiazepines has been attributed to specific α-subunits *(29–31).* Those studies have shown that the anxiolytic actions of benzodiazepines are mediated via α_2-subunit containing $GABA_ARs$.

Although 5-HT lesions and pharmacological enhancement or reduction of 5-HT neurotransmission indicated a role for 5-HT in the control of anxiety *(32),* the evidence for this notion is both conflicting and controversial. Even the development of subtype-specific drugs did not solve the long-standing question of how 5-HT and 5-HT receptors (5-HTRs) control anxiety. On the other hand, pharmacological manipulation of the 5-HT system is definitely an effective way to treat anxiety *(33).* The selective serotonin reuptake inhibitors, first used in depression, have been shown to be very effective in certain anxiety disorders. Also, partial $5\text{-}HT_{1A}R$ agonists, such as buspirone, have potent anxiolytic effect. The beneficial effect of 5-HT drugs, however, by no means implicates 5-HT in the pathogenesis of anxiety disorder.

The role of NE in anxiety reflects the long-standing link between stress, which provokes and aggravates anxiety, and increased catecholamine release by the sympathoadrenal system in the periphery. NE neurons in the locus coeruleus play a critical role in the body's response to alarm and threat, and noradrenergic pathways have long been known to share a close relationship with abnormal states of fear and arousal *(34).* NE is believed to play an important role in disorders such as panic disorder and posttraumatic stress disorder *(35).*

A number of neuropeptides have been implicated in anxiety, and suggested as therapeutic targets *(36).* Disturbed NPY transmission may contribute to the clinical symptoms of anxiety *(37).* In rats, central administration of NPY produced effects similar to that of anxiolytics *(38);* specific inhibition of the NPY-1 receptor by antisense oligonucleotide resulted in an anxiety-like behavior *(39).* Another neuropeptide, CRH, has also been shown to modulate behavioral changes that occur during stress. Central administration of CRH in rodents produces behavioral effects that correlate with a state of anxiety, such as a reduction in exploration of a novel environment, or an enhanced fear response *(40–43).* These effects occur independently of the activation of the hypothalamic-pituitary-adrenal system (HPA), and are considered as the central action of CRH *(42,44).* A similar anxiety-like phenotype has been described in transgenic mice overexpressing CRH *(45).* During the past few years, CCK has emerged as an important polypeptide in the central nervous system (CNS). CCK has been implicated in anxiety, because specific agonists to brain CCK receptors produce anxiogenic-like effects; brain CCK antagonists elicit anxiolytic-like responses in several models of anxiety *(46).* Finally, substance P has also been suggested to have a modulatory role in

anxiety. Substance P has been demonstrated to be released in response to aversive stimuli *(47);* its administration in animal models has elicited both anxiogenic and anxiolytic activity, depending on the dose and the specific brain region *(36).* The receptor for substance P is the G-protein-coupled tachykinin, NK-1 receptor, which is expressed in brain areas associated with fear and anxiety *(48).* Increasing numbers of reports indicate that specific antagonists of NK-1 receptors are anxiolytic *(49,50).* In addition, neonates from NK-1 receptor-null mutant mice emit fewer separation-induced ultrasonic vocalizations, indicating a lower level of anxiety in the absence of the NK-1 receptor *(51).* Taken together, these neuropeptides are important modulators of anxiety-related behaviors, and provide good targets for the development of anxiolytics.

Anxiety-Like Behavior in Knockout Mice

Many components of the neurotransmitter systems implicated in anxiety have been targeted in knockout mouse lines, and shown to have anxiety-like behavior. Table 1 summarizes the results of behavioral tests conducted to assess the level of anxiety in a large number of adult knockout mice. Although the authors have attempted to list all published knockout lines displaying altered anxiety-like behavior, the phenotypes are not always robust, and are sometimes even questionable. Many variables can alter the interpretation of anxiety-related behavioral tests. These tests are not standardized across laboratories, and environmental factors, such as light or sound levels or height of an elevated maze, can affect the outcome. In addition, the level of anxiety in an animal can be influenced by biological factors, which can be controlled for relatively easily (genetic background, sex, age), as well as experiential factors (handling-induced stress, degree of maternal–pup interaction), which are more difficult to control for. Furthermore, anxiety-like behavior may be part of a complex phenotype, and secondary to major developmental or neuroanatomical defects. In particular, if locomotor activity is altered, it is difficult to have confidence in an anxiety-like or anxiolytic-like profile obtained in the EPM or open-field test. Consequently, caution should be used when interpreting the anxiety-like behavior of knockout mice, and the phenotype is more compelling when animals show anxiety in at least two independent behavior tests.

Knockout Mice with Disturbances in Neuronal Messengers Exhibit Anxiety

Neurotransmitters and neuropeptides have long been implicated in anxiety, so it was not surprising that inactivation of genes encoding enzymes responsible for the synthesis and metabolism of neurotransmitters, or the genes encoding neuropeptides, alter anxiety levels.

Glutamic acid decarboxylase (GAD) catalyzes the synthesis of GABA from glutamate, and deletion of the 65-kDa isoform of GAD (GAD65) results in anxiety *(52).* GAD65 is highly expressed in cortex, hippocampus, and cerebellum, where it is associated with nerve terminals and synaptic vesicles, and can be rapidly activated in times of high GABA demand. In *GAD65*$^{-/-}$ tissues, the GABA content is normal, but K^+-stimulated GABA release is reduced. Pharmacological studies showed that modulators that enhance GABA-induced chloride influx (diazepam and pentobarbital) are less effective in *GAD65*$^{-/-}$ mice; the $GABA_AR$ agonist, muscimol, exerts its normal sedative effect. These *GAD65*$^{-/-}$ mice demonstrate that a reduction in readily releasable GABA, independent of any change in function of the postsynaptic $GABA_AR$, can be associated with an anxiety-like phenotype. This indicates that an appropriate level of GABA release is important for maintaining normal behavioral responses in anxiety-inducing situations, and is consistent with reports that enhancing endogenous GABA levels in the rat, e.g., by using GABA reuptake inhibitors, has an anxiolytic effect *(53).*

Table 1
Knockout Mice Exhibiting Altered Anxiety-Like Behavior

Targeted gene/protein	Anxiety behavior test: Elevated maze	Open-field	Light–dark box	Conditioned-fear or potentiated-startle	Locomotor activity	Ref.
Neuronal messengers						
GAD65	+	+			=	Kash et al.*(52)*
COMT			+[a]		=	Gogos et al.*(54)*
NPY	=	+			=	Bannon et al.*(55)*
Enkephalin	+	+			−/=[b]	Konig et al.*(59)*
OFQ/N	+	+	+		−/=[b]	Koster et al.*(62)*
Interferon γ	+	+[c]			=	Kustova et al.*(63)*
NCAM	−		−		+	Stork et al.*(64)*
Midkine	+				=	Nakamura et al.*(66)*
ApoE	+	=/+[d]			=/+[d]	Raber et al.*(67)*
Receptors						
$GABA_AR$ (γ_2)	+		+	=	=	Crestani et al.*(70)*
$5\text{-}HT_{1A}R$	+	+			=	Heisler et al.*(72)*
	+	+			=	Ramboz et al.*(73)*
	+	+			=	Parks et al.*(71)* Sibille et al.*(76)*
$5\text{-}HT_{1B}R$	=	−	=	=	=	Ramboz et al.*(79)* Malleret et al.*(80)*
CRHR1	−		−		=	Smith et al.*(85)*
			−		+	Timpl et al.*(84)*
	−		−		=	Contarino et al.*(86)*
CRHR2	+	+	=		=	Bale et al.*(87)*
	=	=			=	Coste et al.*(89)*
	+	−[e]	+		=	Kishimoto et al.*(88)*
DA D3R	−	−			+	Steiner et al.*(90)*
DA D4R		+			−	Dulawa et al.*(91)*
Mas	+				=	Walther et al.*(92)*
Adenosine A_{2a}	+		+		−	Ledent et al.*(95)*
nAChR (α_7)		−	=	=	=	Paylor et al.*(98)*
nAChR (α_4)	+				=	Ross et al.*(99)*
Intracellular regulators						
Adenylyl cyclase VIII	−	−			=	Schaefer et al.*(103)*
α-CaMKII		−		−		Chen et al.*(106)*
CRE modulator	=/−[f]			=	+	Maldonado et al.*(105)*
PKCγ	−		−		=	Bowers et al.*(109)*
Fyn			+		−	Miyakawa et al.*(112)*
C-term dystrophin			+		−	Vaillend and Ungerer*(116)*
Glucocorticoid receptor	−	−	−		=	Tronche et al.*(119)*
FMRP			−	=	+	Peier et al.*(120)*

A summary of the behavior of adult knockout mice, compared with wild-type, in the EMP or zero maze, the open field, the light–dark box, or conditioning tests, such as fear-potentiated startle and contextual-fear conditioning. Response is indicated as increased anxiety (+), reduced anxiety (–), or no change (=). Note that the degree to which locomotor activity is altered can complicate the interpretation of these anxiety-related behaviors. [a]Gogos et al. *(54)* report an increased anxiety only in female knockout mice. [b]Koster et al. *(62)* and Konig et al. *(59)* report that reduced locomotor activity is evident in the open field, but not the EPM test. [c]Kustova et al. *(63)* used a startle stimulus to elicit an anxiety response in the open field. [d]Raber et al. *(67)* note that the behavior is age-dependent. [e]Kishimoto et al. *(88)* present data indicating increased number of center field visits, which corresponds to a decrease in anxiety, although the authors do not interpret their data in this way. [f]Maldonado et al. *(105)* found no effect of genotype in the EPM, but a decrease in anxiety was seen in the elevated zero-maze.

Inactivation of the enzyme, catechol-O-methyl transferase (COMT), involved in the degradation of DA, NE, and epinephrine, also leads to anxiety *(54).* Measurement of tissue catecholamine levels, in *COMT*$^{-/-}$ mice, showed a specific increase in DA levels, with no change in NE or 5-HT levels. Furthermore, this increase in DA seems to be restricted to the frontal cortex. Although the increased DA levels were evident in both males and females, an increased anxiety behavior was observed only in females. These data are difficult to interpret in terms of a role for DA in anxiety.

The notion that neuropeptides are involved in the pathogenesis of anxiety has been further advanced by mouse knockouts. Mice lacking the gene for NPY show a decrease in central area activity in the open field, and an increased reactivity to acoustic startle *(55).* However, no change in EPM was seen in null mice, compared to wild-type, suggesting that the absence of NPY results in a notable increase in stress responsiveness. Observations of an increased anxiety that is partly reversed by CRH antagonists, in transgenic mice overexpressing NPY, support this idea *(56).* NPY is widely distributed in the CNS, and many of its effects are thought to be related to inhibition of 5-HT and NE release *(57,58).* An inhibitory effect on 5-HT-containing cells, in the dorsal raphe nucleus, could reduce spontaneous firing and consequent hyperpolarization in the locus coeruleus.

Disruption of the preproenkephalin gene led to the generation of enkephalin$^{-/-}$ mice. Konig et al. *(59)* reported that, apart from altered pain responses, these mice exhibit increased anxiety in the open field and elevated zero-maze. A direct role for enkephalins in modulating anxiety has previously been proposed *(60).* More recently, the overexpression of proenkephalin in rats was shown to have no effect on anxiety-related behaviors, although the anxiolytic effects of benzodiazepines were potentiated *(61).* These findings suggest that enkephalins may have a modulatory role on anxiety behavior that is probably mediated via an interaction through the GABA-ergic system.

Genetic inactivation of the neuropeptide, orphanin FQ/nociceptin (OFQ/N), also results in increased anxiety *(62).* In the open field test, *OFQ/N*$^{-/-}$ mice exhibited reduced activity, and spent less time in the center of the field, spent more time in the dark in the light – dark box, and made fewer entries into the open arms of the EPM. OFQ/N is expressed in brain areas involved in the processing of stress responses and anxiety-related behavior, such as the hypothalamus, hippocampus, and amygdala. Increased plasma corticosterone levels, and a failure to show stress adaptation, accompany the anxiety-related behavior of OFQ/N knockout mice, suggesting that activation of the HPA underlies the anxiety phenotype in these mice.

Besides neurotransmitters and neuropeptides, there are other molecules in the brain involved in neuronal communication, whose deletion leads to an anxiety-like phenotype. Lack of the cytokine protein, interferon γ, has been reported to cause an anxiety-like phenotype *(63).* However, the expression of this phenotype was visible only in C57Bl6, but not in the Balb/c mouse strain, indicating that major genetic modifiers play a role in the manifestation of anxiety. Interferon γ is involved in regulating the growth of axodendritic processes, raising the possibility that a neurodevelopmental abnormality underlies the anxiety in these knockout mice. Similarly, genetic inactivation of the neuronal cell adhesion molecule (NCAM), a protein involved in cell–extracellular matrix interactions, as well as in signal transduction, results in decreased anxiety in the light–dark and EPM tests *(64).* In addition, these mice responded to the anxiolytic effect of buspirone at lower doses than the *NCAM*$^{+/+}$ mice, suggesting that there may be an alteration in the sensitivity of the 5-HT_{1A}Rs in these knockout mice. However, those authors reported no changes in the density of 5-HT_{1A}Rs or in tissue 5-HT content. Because NCAM has been demonstrated to have roles in CNS development and neuroplasticity processes, a developmental abnormality may explain the appearance of

anxiety-like behavior in these mice. Another factor with an important role in neuorogenesis, cell migration, and mesoderm-epithelial interactions, is the heparin-binding growth factor, midkine *(65).* Deletion of midkine results in increased anxiety and impaired short-term memory in 4-wk-old mice, but this phenotype disappeared by 8-wk of age *(66).* Since midkine$^{-/-}$ mice have increased calretinin immunoreactivity in dentate gyrus and cortex during postnatal development, those authors suggested that, in the knockout mice, some developmentally regulated genes are altered, which cause a temporally delayed, but otherwise normal, development of the dentate gyrus and cortex. Finally, Raber et al. *(67)* reported that apolipoprotein E (apoE)-deficient mice display anxiety in the EPM. ApoE is the major apolipoprotein in the cerebrospinal fluid, and has been implicated in Alzheimer's disease. Apart from being a lipid transporter molecule, apoE has multiple functions, including a neurotrophic and neuroprotective effect *(68,69).* Since *apoE$^{-/-}$ mice* also display age-dependent differences in stress-induced adrenocorticotropic hormone release, corticosterone release, and adrenal corticosterone content, Raber et al. (67) suggested that the observed anxiety phenotype of apoE-deficient mice is related primarily to the peripheral effects of apoE on the HPA axis. The authors did not address the possibility that the anxiety phenotype of apoE knockout mice might be associated with the loss of the neuroprotective function of apoE. Taken together, these reports have identified cytokines/growth factors as being associated with anxiety-related behavior.

Knockout Mice with Deficits in Neurotransmitter Receptors Display Anxiety

Genetic disruption of the $GABA_AR$, target of the benzodiazepines, was expected to elicit anxiety. However, the $GABA_AR$ is a pentameric ion channel, typically composed of 2α (α_{1-6}), 2β (β_{1-3}) and 1γ (γ_{1-3}) subunits, and it was not known which of the subunits was the most important determinant of anxiety. Essrich et al. *(28)* reported that inactivation of the gene encoding the γ_2-subunit of the $GABA_AR$ results in anxiety in mice. The γ_2-subunit is required for normal single-channel conductance and synaptic clustering *(28),* and is incorporated in most $GABA_ARs$. $\gamma_2^{-/-}$ mice have a large number of functionally impaired receptor channels, and die around birth. However, heterozygote ($\gamma_2^{+/-}$) mice survive, but have reduced numbers of $GABA_ARs$ and display an anxiety phenotype *(28,70).* In cortex, hippocampus, and thalamus of the $\gamma_2^{+/-}$ mice, electrophysiological studies demonstrated a lower single-channel conductance, as well as a pronounced deficit of functional receptors; immunological studies revealed a reduction in α_2/gephyrin containing postsynaptic $GABA_AR$ clusters. The $\gamma_2^{+/-}$ mouse spends less time in the open arms of the EPM, and less time in the lit area of the light–dark box, typical of increased anxiety-type behavior. In addition, the $\gamma_2^{+/-}$ mice showed an increased responsiveness in the passive-avoidance protocol, which is consistent with emotional memory for negative associations, a common feature of several anxiety disorders. Whether disruption of other subunits of the $GABA_AR$ complex is associated with anxiety remains to be determined.

Besides the $GABA_AR$, the $5\text{-}HT_{1A}R$ has been frequently implicated in the pathogenesis of anxiety disorders. In 1998, three groups reported the generation of a $5\text{-}HT_{1A}R$ knockout mouse on different strain backgrounds *(71–73).* All three groups reported that the mutant mice demonstrate consistently enhanced anxiety-type behaviors alongside reduced immobility in the forced-swim test *(71)* or tail-suspension test *(72,73),* indicating an antidepressant-like effect. $5\text{-}HT_{1A}Rs$ are expressed at postsynaptic locations in 5-HT target areas (such as amygdala, hippocampus, and cortex), and also on 5-HT neurons in the raphe nuclei as somatodendritic autoreceptors. Therefore, there was a possibility that anxiety in the $5\text{-}HT_{1A}R$ knockout mice was caused by an increase in 5-HT release and acti-

vation of other 5-HTR subtypes. However, expressing 5-HT_{1A}Rs at postsynaptic sites in forebrain regions rescues the phenotype of 5-HT_{1A}R knockout mice *(74).* Thus, it seems likely that the phenotype results from the absence of postsynaptic 5-HT_{1A}Rs and inactivation of the associated signaling cascade. A surprising consequence of the absence of 5-HT_{1A}Rs is an altered regulation of the GABA system. In the cortex, amygdala, and hippocampus of 5-$HT_{1A}R^{-/-}$ mice, there are α-subunit-specific changes in $GABA_AR$ composition *(75).* This suggests the existence of a novel mechanism whereby an abnormality in one neurotransmitter receptor might cause anxiety by the misregulation of a different transmitter receptor.

Another member of the 5-HTR family, whose deletion has been associated with an alteration in anxiety, is the 5-HT_{1B}R. 5-HT_{1B}Rs are predominantly localized to nerve terminals, and serve as both auto- and heteroreceptors to inhibit neurotransmitter release *(76,77).* Initially, the principal behavioral phenotype of the 5-HT_{1B}R knockout mouse was reported to be an increased isolation-induced aggression, thought to be related to an alteration in the regulation of release of 5-HT and other transmitters *(78,79).* The light–dark box test and the more anxiogenic EPM test showed no significant anxiolytic response in the 5-HT_{1B}R knockout, although the open field test indicated some reduced anxiety-like behavior *(80).* Brunner et al. *(81)* examined the reciprocal effects of maternal–pup interaction on anxiety in the 5-HT_{1B}R knockout mice. The knockout pups exhibited less isolation-induced ultrasonic vocalization than age-matched, wild-type pups, which suggests that the 5-HT_{1B}R has an important role in the development of an anxiety phenotype, which is not readily apparent in adult animals.

There are two known corticotrophin-releasing hormone receptors (CRHR 1 and 2), and both have been suggested to be important in regulating anxiety levels. The ligand for both receptors is CRH, a potent mediator of endocrine, autonomic, behavioral, and immune responses to stress. A second endogenous ligand for CRHRs has been identified, the neuropeptide, urocortin, which has anxiogenic properties *(82,83).* CRHR1 has a widespread distribution with high levels in anterior pituitary, hippocampus, amygdala, and cerebellum. Although the receptor in the anterior pituitary is involved in the activation of the HPA, receptors in other regions are responsible for the central action, i.e., anxiogenic effect, of CRH. Mice lacking CRHR1 display decreased anxiety in the light–dark box and the EPM *(84–86).* In contrast to CRHR1, expression of CRHR2 in the CNS is restricted to the lateral septum and the ventromedial nucleus of the hypothalamus. The $CRHR2^{-/-}$ mouse, generated independently by three groups, exhibits varying degrees of anxiety-related behavior. Increased anxiety was evident in the EPM and open field, but not in the light–dark box test *(87).* In the study of Kishimoto et al. *(88),* only male $CRHR2^{-/-}$ mice exhibited anxious behavior in EPM and in light–dark box, but, paradoxically, spent more time in the center of the open field, which is consistent with a reduced anxiety. In the third group of $CRHR2^{-/-}$ mice, no significant change was seen in anxiety parameters in the EPM or open field *(89).* Taken together, the studies of CRHR knockout mice indicate that CRH or urocortin mediate a reciprocal modulation of anxiety behavior. Activation of CRHR1 appears to be anxiogenic; activation of CRHR2 is anxiolytic. However, the precise interaction between these components requires further investigation, particularly in view of the uncertain phenotype of the CRHR2 knockout mouse.

A possible modulatory role of DA in anxiety-related behaviors has previously been suggested, because agonists and antagonists for the DA D2 class of receptors (including D2, D3, and D4 subtypes) have anxiogenic and anxiolytic properties, respectively. The DA D3 receptor (D3R) knockout mouse displays reduced anxiety in the open field and EPM, associated with increased locomotor activity *(90).* In contrast, mice with the $D4R^{-/-}$ genotype exhibit enhanced anxiety in the open field test, in the presence of a novel object *(91).* The response in this variation of the open field test involves a complex behavior, a conflict between the interest in

exploring the novel object and the fear/anxiety of the novel environment. Altered anxiety levels were evident, but those authors ascribed much of the behavioral phenotype to changes in exploratory behavior, rather than to an anxiety-based behavior *per se.*

Anxiety has also been reported in animals with the deletion of several other receptors (*mas* proto-oncogene, adenosine$_{2a}$ (A_{2a}) receptor, and nicotinic acetylcholine receptors [nAChRs]), whose involvement in anxiety is unclear. Because of limited behavioral testing, conflicting behavioral findings, and/or limited information on the receptor themselves, it is not clear how to explain the manifestation of anxiety in these knockout mice, and whether these receptor-knockout mice have a genuine anxiety phenotype. Walther et al. *(92)* reported that genetic inactivation of the *mas* proto-oncogene, a G-protein-coupled receptor, results in increased anxiety in the EPM-test. *mas* is expressed in hippocampal neurons, cortex, and amygdala, and its putative ligands are angiotensin II and III. Although some evidence suggests that angiotensin II is anxiogenic *(93),* the angiotensin II knockout has no behavioral abnormalities *(94).* Adenosine$_{2a}$ (A_{2a}R) mice show increased anxiety in elevated maze and light–dark box, but also display decreased exploratory behavior and decreased locomotor activity, which could confound the anxiety phenotype *(95).* Nevertheless, the anxiety of the knockout mice is consistent with the finding that adenosine has calming effects, but caffeine, a nonspecific antagonist at adenosine receptors, causes anxiety in animals *(96).* The A_{2a} receptor is co-expressed with DA D2Rs in GABA-ergic neurons in basal ganglia and striatum, and is thought to regulate the expression of the proenkephalin gene *(97). In situ* hybridization studies showed decreases in both proenkephalin and protachykinin gene expression in the A_{2a} receptor-knockout mice, which may underlie the anxiety-like behavior. Mice with a null mutation in the *nAChR α_7-subunit* gene exhibit decreased anxiety in the open field, but little difference in behavior in the light–dark box *(98).* In contrast, mice deficient in the *nAChR α_4-subunit* gene display an increased anxiety in the elevated maze test *(99).* Nicotinic agonist and antagonists can modulate anxiety *(100,101),* and it seems that the subunit composition of the nAChR may determine whether the effect is anxiogenic or anxiolytic.

Intracellular Regulators Associated with Anxiety-Like Phenotype

Genetic inactivation of intracellular signaling molecules and regulators of gene expression have been shown to cause an anxiety phenotype.

Adenylyl cyclase type VIII is an enzyme that is stimulated by calcium and calmodulin to produce cyclic adenosine monophosphate (cAMP). This isoform is expressed at high levels in the hippocampus and in cells involved in the neuroendocrine response to stress *(102).* In a single naive trial, in either the EPM or open field, there was no difference between wild-type and adenylyl cyclase VIII knockout animals *(103).* However, wild-type mice showed increased anxiety behavior on repeated exposure to the test environment, which acts as a chronic stressor. The adenylyl cyclase VIII knockout animals did not experience anxiety in repeated trials, suggesting that adenylyl cyclase VIII has a role in the attenuation of stress-induced anxiety. A family of transcription factors binding to cAMP-responsive elements (CREs) is phosphorylated in response to cAMP *(104).* The phosphorylation of one of these factors, CRE-binding protein, is disrupted in the hippocampus of adenylyl cyclase VIII knockout mice *(103).* This suggests that inactivation of specific signaling molecules could lead to changes in gene transcription, which in turn would lead to anxiety phenotype. CRE modulator protein (CREM), a factor related to CRE-binding protein function, has also been implicated in anxiety. The CREM gene generates several products in a tissue-specific manner, which may activate or inhibit cAMP-responsive genes. CREM is involved in the control of various neuroendocrine

responses, and is thought to have an important role in regulation of circadian rhythms. A decrease in anxiety-like behavior is evident in *CREM*$^{-/-}$ mice *(105).* However, CREM-deficient mice are hyperactive, and do not show the characteristic day–night change in locomotion, demonstrating that the anxiety-related behavior is just one aspect of a more complex phenotype.

Chen et al. *(106)* demonstrated a decreased anxiety in mice deficient in another important second messenger, the α-isoform of calcium/calmodulin-dependent protein kinase II (CaMKII). CaMKII is a major component of the postsynaptic density *(107)* in glutamatergic synapses, and is involved in neuronal function, particularly related to calcium signaling, including the induction of long-term potentiation *(108).* Therefore, a disruption in normal CaMKII function could alter many aspects of neuronal function, and it is difficult to relate this to a specific anxiety behavior; indeed, these knockout mice also exhibit enhanced aggression and learning impairment.

The serine/threonine kinase, protein kinase C γ (PKCγ), has recently been shown to be a regulator of anxiety behaviors *(109).* PKCγ is restricted to the CNS, and is highly expressed in limbic areas of the brain. In three different behavioral tests, PKCγ knockout mice consistently showed reduced anxiety. Bowers et al. *(109)* proposed that PKCγ modulates anxiety by altering the function of $GABA_A$, N-methyl-D-aspartate, or 5-HT_2 receptors. Phosphorylation of the $GABA_AR$ γ_{1L}-subunit, by PKC is necessary for the ethanol-induced potentiation of $GABA_AR$ responses *(110).* PKCγ knockout mice have reduced sensitivity to ethanol, although they do not show altered responses to benzodiazepines *(111).* Further studies are required to understand how the absence of PKCγ could alter the function of $GABA_AR$ to produce a reduced-anxiety phenotype.

Another intracellular signaling molecule, implicated in anxiety and fear responses, is the tyrosine kinase, fyn. Fyn is a member of the Src family of tyrosine kinases that can associate with and phosphorylate a variety of molecules. Inactivation of the *fyn* gene results in mice that exhibit increased anxiety to naturally aversive stimuli in the light–dark box and novelty tests *(112).* These mice also displayed enhanced learned-fear responses in the passive-avoidance test. Fyn is highly expressed in the limbic system, and has been implicated in N-methyl-D-aspartate receptor-mediated synaptic plasticity, NCAM-dependent neurite outgrowth, and myelination *(113–115).* Whether any or all of these processes is involved in the enhanced anxiety exhibited in the *fyn*$^{-/-}$ mice is unclear.

C-terminal dystrophins are cytoskeletal proteins that are widely expressed in the brain, where they form complexes with both cytoplasmic and integral membrane proteins. The mdx3cv mouse has a dramatic reduction of the C-terminal dystrophins, and these mice display increased anxiety in the light–dark box, slightly reduced locomotor activity, and little evidence of learning impairment *(116).* One function proposed for dystrophin is that, as part of the dystrophin protein complex, it influences intracellular signaling in neurons *(117).* Impairment in intracellular signaling could underlie the anxiety-related behavior observed in the mdx3cv mice.

Direct regulation of gene expression has been revealed as a novel process involved in anxiogenesis by inactivation of the glucocorticoid receptor (GR) and *fmr1* genes. GR is an intracellular receptor that interacts with glucocorticoids in the cytoplasm. Activated receptors enter the nucleus and regulate the transcription of target genes, such as CRH, directly, by interaction with DNA-regulatory elements, and indirectly, by crosstalk with other transcription factors *(118).* Hippocampus and amygdala have a high density of GRs, but the specific function of the receptors in these regions could not be studied until a region-specific knockout mouse line was generated *(119).* In contrast to the lethal phenotype seen in mice with an overall receptor inactivation, loss of the GRs in hippocampus results in reduced anxiety-related behaviors in the light–dark box, and in the elevated zero-maze. The anxiety phenotype of mice with a hippocampal-specific loss of GR is in good agree-

ment with the notion that increased glucocorticoid levels, such as occurs during prolonged stress or chronic activation of the HPA, can elicit anxiety. Furthermore, these data suggest that the hippocampus is important in the glucocorticoid-mediated modulation of anxiety states.

Inactivation of the *fmr-1* gene results in reduced anxiety in both open field and light–dark box in mice *(120).* Methylation-induced inactivation of the *FMR-1* gene in human causes fragile X syndrome, the most common form of mental retardation *(121).* Fragile X is often associated with anxiety, and it is not clear why the genetic inactivation of the gene in mouse results in a reduced anxiety. Nevertheless, consistent with the notion that the *fmr-1* gene product (Fragile X mental retardation protein [FMRP]) can influence anxiety-like behavior in mice, transgenic overexpression of the *fmr-1* gene leads to anxiety *(120).* FMRP is an RNA-binding protein involved in mRNA transport and processing, as well as translation. A small fraction of the brain mRNAs are estimated to be targets for FMRP. Although the precise nature of the mRNAs involved, and the defect in mRNA processing, is not clear, it is known that loss of FMRP leads to abnormalities in neuronal morphology and plasticity *(122,123).*

Taken together, defects in several intracellular signaling molecules, including second messengers, transcription factors, and proteins involved in RNA processing, can lead to alterations in anxiety in mice. A common feature of these molecules is that they all eventually influence morphological and/or functional plasticity in the nervous system.

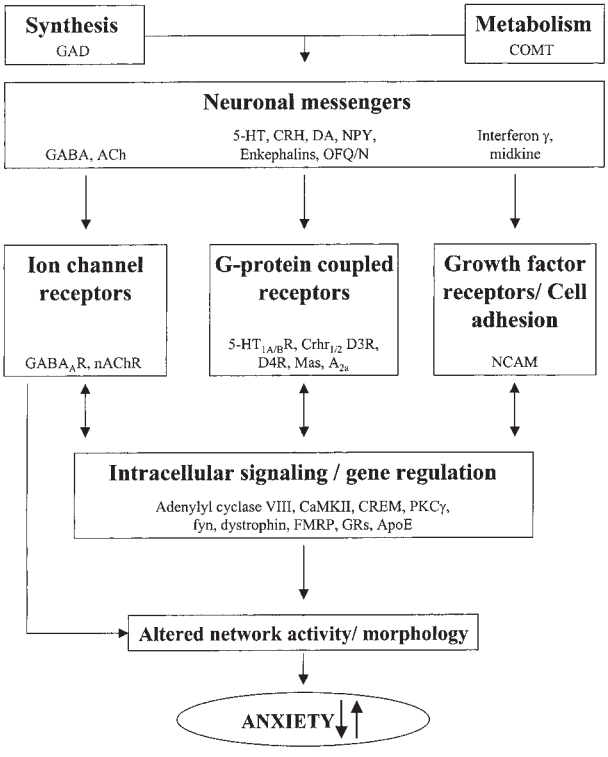

Fig. 1. Knockout mouse strains reveal that neuronal communication, intracellular signaling, and gene expression are involved in anxiety-related behavior. A unifying concept in the pathogenesis of anxiety may be drawn, namely, extracellular neuronal messengers, and their associated receptors and intracellular signaling cascades, represent vulnerable pathways in anxiety. Abnormalities in these pathways can directly modify neuronal function, and cause an anxiety phenotype. Since the receptors and signaling cascades are coupled to gene regulation, defects in neuronal communication can also alter gene expression and indirectly cause long-term changes in neurons. Consequently, neuronal development, morphology, network connectivity, and function are altered, leading to anxiety-related behavior.

Neuronal Communication and Regulation of Intracellular Signaling and Gene Expression are Vulnerable Processes in Anxiety

Surveying the diverse knockout mouse strains that exhibit alterations in anxiety, the affected molecules are apparently either involved in communication between neurons or as regulators of intracellular signaling and gene expression (Fig. 1). Based on pharmacological studies, it was not surprising that deletion of proteins involved in neurotransmitter

biogenesis (GAD65) or inactivation (COMT), neuropeptides (NPY, enkephalin, OFQ/N), and receptors ($GABA_AR$, $5\text{-}HT_{1A/B}R$, CRHR1/2, DA D3/4R, nAChR) would alter anxiety-related behaviors. On the other hand, a number of proteins that have not previously been implicated in anxiety disorders have been identified, using knockout mice. These include several molecules with neurotrophic-type effects, which have important roles in neurite outgrowth and neuronal development (interferon γ, NCAM, midkine, ApoE). This raises the possibility that anxiety disorders might arise as defects in developmental processes. Perhaps the most interesting group of molecules that has been identified is the direct and indirect intracellular regulators of gene expression (adenylyl cyclase VIII, CaMKII, CREM, PKCγ, Fyn, GRs, FMRP). Although the fact that extracellular signals are linked to intracellular pathways is common knowledge, a defect in intracellular signaling or gene expression *per se* has not previously been recognized as a potential cause for anxiety disorders.

One advantage of using gene inactivation in mouse strains to study anxiety has been the ability to answer questions that could not be addressed using pharmacological approaches. For example, many 5-HT receptor agonists and antagonists show a lack of specificity between multiple receptor subtypes. Genetic inactivation of the $5\text{-}HT_{1A/1B}$Rs has helped to elucidate the specific roles of these receptors in anxiety. Similarly, dissecting the role of the two CRH receptors, CRHR1 and 2, has been made easier by the advent of knockout technology. A reciprocal relationship seems likely to exist whereby activation of CRHR1 receptors is anxiogenic, but activation of CRHR2 receptors is anxiolytic. In addition, the importance of the subunit composition of an ion channel receptor is revealed, using molecular approaches. Of particular interest is the $GABA_AR$, since it is the site of action of benzodiazepines, and much interest has focused recently on identifying the α-subunit responsible for the anxiolytic vs sedative effects of these drugs *(29–31).* Although there have been no reports of $GABA_AR$ α-subunit knockouts linked to anxiety, the γ_2-subunit has a clear anxiety phenotype *(70).* Similarly, subunit-specific knockouts of neuronal nAChRs revealed different anxiety behaviors, highlighting the importance of the subunit composition for anxiolytic/anxiogenic actions of nicotinic agonists and antagonists. These advances make possible the design of more specific therapeutics for the treatment of anxiety.

Given that the anxiety-related pathways outlined in Fig. 1 are common to many neurons, one might expect a significant crosstalk between different components. Such crosstalk has been reported to occur in the $5\text{-}HT_{1A}R$ knockout mice. An impaired GABA-ergic transmission, based on altered $GABA_AR$ subunit composition, has been suggested to contribute to the anxiety phenotype *(75).* Complex interactions have also been reported between serotoninergic and HPA systems. Products of the HPA axis modulate serotonergic transmission and 5-HT stimulates the activity of the HPA axis at all levels, mainly through $5\text{-}HT_{1A}$Rs and $5\text{-}HT_2$Rs *(124–126).* Since several of the human anxiety disorders have common characteristics, such interactions at a molecular level may contribute to a degree of overlap between anxiety phenotypes.

The phenotype is not always robust, but knockout technology has revealed several novel anxiety-related molecules, and provided animal models in which to study the complex processes underlying anxiety. Defects in neuronal messengers and regulators of intracellular signaling and gene expression are likely to lead to anxiety-like behavior, by disrupting neuronal development, synaptic function, neuronal connectivity, and/or network function. Furthermore, these intracellular molecules represent potential targets for designing novel therapeutic approaches to the treatment of anxiety. In the future, it will be interesting to see whether intracellular signaling and gene regulation are revealed as vulnerable processes in patients with anxiety disorders.

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