

REVIEW

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Anxiety genetics – findings from cross-species genome-wide approaches

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Abstract

Anxiety disorders are complex diseases, which often occur in combination with major depression, alcohol use disorder, or general medical conditions. Anxiety disorders were the most common mental disorders within the EU states in 2010 with 14% prevalence. Anxiety disorders are triggered by environmental factors in genetically susceptible individuals, and therefore genetic research offers a great route to unravel molecular basis of these diseases. As anxiety is an evolutionarily conserved response, mouse models can be used to carry out genome-wide searches for specific genes in a setting that controls for the environmental factors. In this review, we discuss translational approaches that aim to bridge results from unbiased genome-wide screens using mouse models to anxiety disorders in humans. Several methods, such as quantitative trait locus mapping, gene expression profiling, and proteomics, have been used in various mouse models of anxiety to identify genes that regulate anxiety or play a role in maintaining pathological anxiety. We first discuss briefly the evolutionary background of anxiety, which justifies cross-species approaches. We then describe how several genes have been identified through genome-wide methods in mouse models and subsequently investigated in human anxiety disorder samples as candidate genes. These studies have led to the identification of completely novel biological pathways that regulate anxiety in mice and humans, and that can be further investigated as targets for therapy.

Keywords: Anxiety disorders, Anxiety-like behavior, Mouse model, Cross-species approach, Genome-wide association study, Quantitative trait locus, Gene expression, Proteomics, Candidate gene

Review

Anxiety disorders

Anxiety and fear are normal emotional responses to threatening situations. In anxiety disorders these responses are exaggerated or prolonged and disturb daily life. Anxiety disorders, including panic disorder, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), social phobia, specific phobias, and generalized anxiety disorder (GAD), were the most common mental disorders within the EU states in 2010 with 14% prevalence [1]. Anxiety disorders are currently treated with drugs and/or cognitive behavioral therapy or other psychosocial treatments. Current pharmacotherapeutic options including benzodiazepines and selective serotonin reuptake inhibitors are not optimal due to addictive properties, development of tolerance, or poor efficacy in some patients. Therefore, new

and better anxiolytics are needed, and their development requires understanding of the molecular mechanisms that regulate anxiety. Genetics offer an ideal route to the molecular background of anxiety as any identified genes can directly be linked to their function within the cell and the neural circuits.

Anxiety disorders are complex diseases caused by a combination of genetic and environmental factors. In recent years, several genes have been associated with anxiety disorders [2]. Replicated associations exist to genes belonging to various neurotransmitter or neuropeptide systems [3]. Recently, the first genome-wide association studies (GWAS) aiming to identify common variants have been published in anxiety-related personality trait neuroticism and panic disorder [4-7]. These studies support involvement of a relatively large number of small effect size common and rare variants in the predisposition to anxiety disorders, a notion shared with other psychiatric diseases, such as schizophrenia and major depression. Therefore, very large sample sizes (several thousands of individuals)

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will be needed to identify variants predisposing to anxiety disorders.

Anxiety is an evolutionarily conserved response and can be reliably measured in mice (Table 1). The advantage of mouse models is that the environmental factors can be controlled for, or specifically administered. In addition, brain tissue can be collected at any time point. To complement human genetic studies several groups have used mouse models of anxiety-like behavior for identification of genes and biological pathways that regulate anxiety. In general two approaches can be taken: i) candidate gene studies have mostly used transgenic models to investigate a role of a specific gene in the regulation of anxiety, and ii) genome-wide approaches do not make any prior assumptions regarding which genes contribute to the phenotype. In this review, we will concentrate on genome-wide approaches in mice, which have resulted in the identification of genes regulating anxiety. We have further restricted our focus to those genes that have subsequently been associated at some level to human anxiety disorders. Therefore, several interesting genes that may regulate anxiety but i) have been identified initially through transgenic mouse models, ii) human candidate gene or GWAS studies, or iii) have been identified in mouse models but not shown any link to human anxiety disorders, are not discussed here [8-10].

Anxiety is an evolutionarily conserved response

Why can we use the mouse to model aspects of human anxiety disorders? Neuroevolutionary studies have shown that anxiety is an adaptive response that has been conserved during evolution [12,13]. From this perspective anxiety is viewed as a behavioral state, which occurs in response to signals of danger. On the physiological level these signals initiate activation of the hypothalamus-pituitary-adrenal (HPA) axis [14] and secretion of adrenal steroids called stress hormones, which are present in almost every vertebrate cell [15]. This leads to increased heart rate,

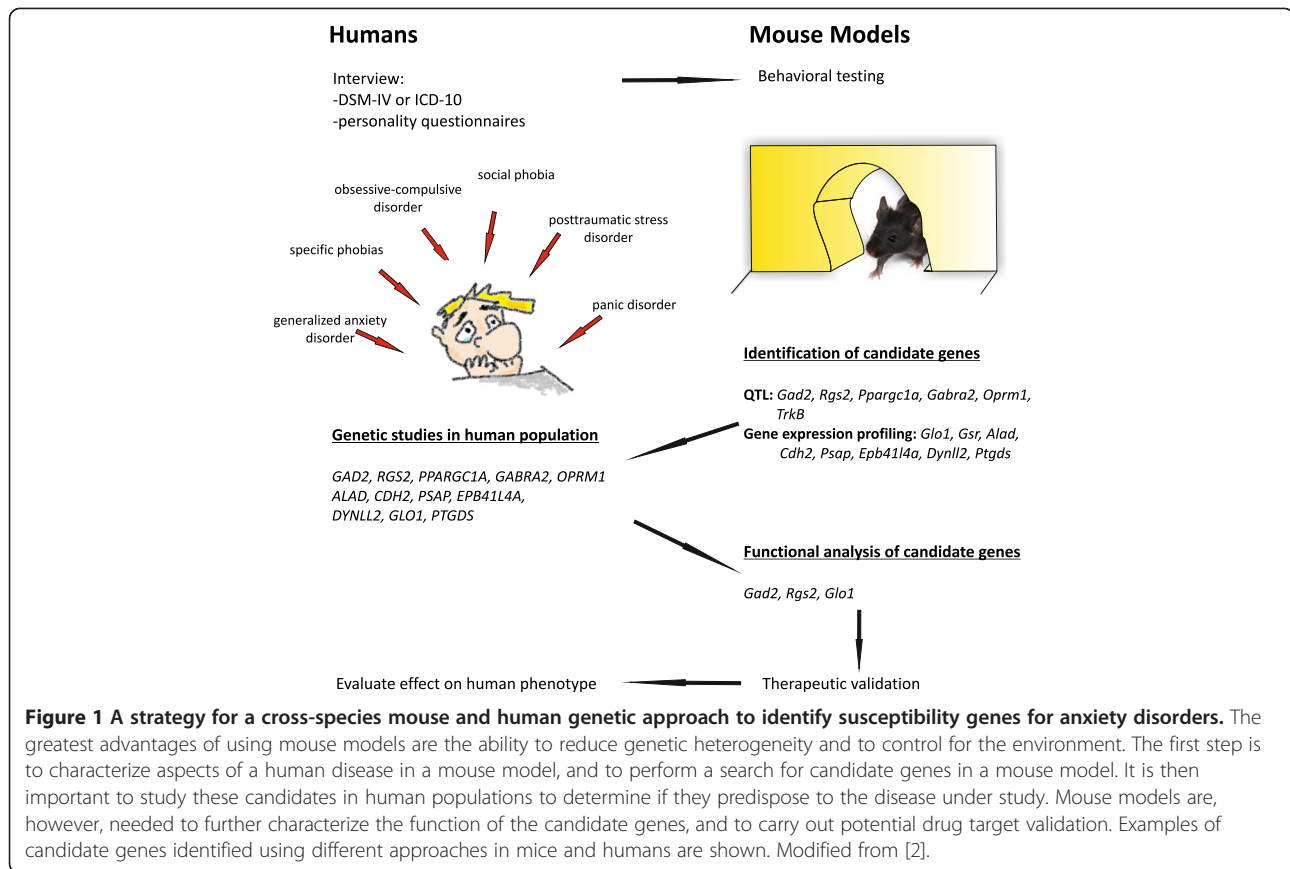
deeper breathing, vigilance, decrease in feeding, and exploration of environment [16]. The genes that code for stress hormones are highly conserved across diverse species: primates, rodents, reptiles, and amphibians [17,18].

Mice represent a good model system for human anxiety disorders for several reasons: i) they have a central nervous system (CNS) that is sufficiently developed to model aspects of human anxiety as compared to lower organisms, ii) hundreds of inbred strains are available, and the whole genome sequence of 17 strains has been determined [19], iii) transgenic techniques to manipulate the genome are well established, and iv) their maintenance is cost-effective. The majority of the anxiety-related behavioral tests utilize approach-avoidance behaviors that appear to mirror rodent's behavioral response to conflict in its natural environment. Both approach behaviors, such as mate searching and foraging, and avoidance behaviors, such as escape from the predator, are evolutionarily conserved in some forms from nematodes to mammals [20]. Furthermore, the neural organization of behaviors underlying fearful, sexual, feeding, and escape motivation is relatively similar across species [21]. Disturbed balance in approach-avoidance behaviors is a symptom of autism [22], PTSD [23], and social phobia [24]. Several paradigms to test anxiety in mice, based on the approach-avoidance behavior, have been developed and pharmacologically validated with drugs that are used to treat human disease and are therefore considered appropriate models for human anxiety [25]. The most commonly used tests include the elevated plus maze, open field, light dark box, and novelty-induced hypophagia tests. In these tests mice have to choose between exploring and staying in a safe environment. However, due to cognitive differences between mouse and human, it is recognized that no animal model can mimic all aspects of human anxiety and anxiety disorders. Nevertheless, genes that regulate anxiety in mice are excellent candidate genes for anxiety disorders (Figure 1).

Table 1 Comparison of human anxiety disorders to anxiety-like behavior in mice

Disorder	Human symptoms	Observed behavior in mice	Behavioral test in mice
Generalized anxiety disorder	Excessive worry about everyday life, leading to difficulties in concentration	Decreased social interaction, impaired sustained attention	OF, L/D, Y-maze
Posttraumatic stress disorder	Repeated re-experiencing traumatic events, leading to avoidance of stimuli associated with trauma	Increased freezing response to fear conditioning, decreased fear extinction, more pronounced spontaneous recovery	Cue and contextual fear conditioning, fear extinction
Obsessive-compulsive disorder	Intrusive thoughts that produce repetitive behavior aimed at reducing anxiety	Increased marble burying and excessive grooming	Burrowing test, nest construction test
Social phobia	avoidance of social contact, emotional discomfort caused by presence of unknown people	Low social interaction	Three-chamber test of sociability, social recognition test
Panic disorder	Intense fearfulness of sudden onset, respiratory distress	Increased escape from an aggressor	Resident intruder test
Agoraphobia	Avoidance of wide-open or crowded space	Avoidance of exposed, bright areas	OF, L/D

Anxiety disorders are classified according to the Diagnostic and Statistical Manual of Mental Disorders of American Psychiatric Association (DSM-IV). OF, Open field test; L/D, Light/dark box test. Modified from [11].



Quantitative trait locus (QTL) mapping of anxiety-like behavior

QTL mapping has been used to identify genes that regulate anxiety-like behavior in rodents [26], with the idea that genes in the homologous loci in humans can then be studied as susceptibility genes for the corresponding human phenotype. Traditionally, QTL mapping has been based on genotyping F2 mice using a genome-wide marker panel and measuring the anxiety level of these animals. As a result, loci that likely contain genes affecting the phenotype can be mapped. Due to the low mapping resolution of F2 panels, other sources, such as recombinant inbred strains, heterogeneous stock mice, and outbred animals, have been used for initial and fine mapping [27-29]. It is expected that the Collaborative Cross strains, a collection of recombinant inbred mouse strains derived from eight parental strains, will be an efficient mapping resource in the future to identify both major loci and their modifiers [30]. Although initial enthusiasm for QTL mapping has been suppressed by low efficiency and resolution, and small effect size of individual variants, several anxiety-associated genes have been identified through QTL mapping [31-37]. Here we will discuss those genes that have shown some evidence for association

to human anxiety disorders in subsequent studies. These include *Gad2*, *Rgs2*, *Ppargc1a*, *Gabra2*, *Oprm1*, and *TrkB*.

Glutamic acid decarboxylase 2 (*Gad2*)

One of the earliest cross-species studies investigated behavioral inhibition to the unfamiliar, a heritable temperament character that is considered a risk factor for panic and phobic anxiety [38]. Four genes were selected for genotyping in humans based on their homology to loci previously associated with anxiety or fear behavior in mice. The sample consisted of 72 behaviorally inhibited children and their family members, analyzed in a family-based association analysis. Suggestive evidence for association was found to variants in the *GAD2* gene. *GAD2* is an enzyme involved in the gamma-aminobutyric acid (GABA) synthesis, and is therefore an intriguing candidate gene as abnormalities in the GABA system have been observed in anxiety disorders [39]. *GAD2* has been studied as a candidate gene for anxiety disorders in two larger subsequent studies. In the Virginia Adult Twin Study of Psychiatric and Substance Use Disorders 14 SNPs from *GAD2* were first genotyped in 188 cases with internalizing disorders (major depression, GAD, panic disorder, agoraphobia, social phobia, or neuroticism personality trait) and

188 controls. One SNP with $p < 0.1$ and two SNPs within the same haplotype were followed up by genotyping additional 401 cases and 351 controls but the initial finding was not replicated [40]. Another study, consisting of anxiety disorder cases ($N = 268$), cases with major depression ($N = 541$), and 541 healthy controls, tested association to 18 SNPs within *GAD2* [41]. One SNP (rs8190646) significantly associated to major depression ($p = 0.00039$). No association to anxiety disorders was found. To mimic the phenotype of the original study [38] association of these SNPs were also tested with behavioral inhibition in 211 anxiety disorder cases, 202 cases with major depression, and 537 controls from the same sample. Significant association to behavioral inhibition was found in the subgroups of anxiety disorder cases and healthy controls, but not to cases with major depression or all groups combined. The contradictory findings in these two larger studies may be explained by several differences, such as phenotype definition and ethnicity of the study participants. The large ongoing GWAS studies should inform whether *GAD2* variants predispose to various anxiety disorders or other psychiatric phenotypes.

Regulator of G-protein signaling 2 (*Rgs2*)

A more recent successful cross-species study concerns the genetic background of emotionality. Initially, a linkage to chromosome 1 was found by QTL mapping of DeFries mouse strains [42], and the locus was fine mapped in outbred mice [43]. This region contains the *Rgs2* gene, encoding a regulator of G protein signaling. To investigate whether *Rgs2* interacts with the functional variant, quantitative complementation method was applied, and a small-effect QTL contributing to behavioral variation in mice was identified [44]. Furthermore, knock-out mice of *Rgs2* show increased anxiety-like behavior [45]. These results indicate that *Rgs2* regulate anxiety-like behavior in mice. To study the involvement of variants in *RGS2* in intermediate phenotypes of human anxiety disorders Smoller et al. studied a family based sample (119 families) of children with behavioral inhibition, 744 unrelated adults who were tested for extraversion and introversion personality traits, and 55 unrelated adults tested with the emotional face assessment during fMRI [46]. *RGS2* SNPs associated with childhood behavioral inhibition (haplotype $p = 0.00003$) and introversion personality trait ($p = 0.007-0.05$ for single SNPs, $p = 0.038$ for a haplotype) as well as increased activation of amygdala and insular cortex in response to watching fearful faces. In another study, four SNPs within *RGS2* showed some association to panic disorder ($p = 0.02-0.05$) in a sample of 173 German cases and 173 controls [47]. Also, one SNP in *RGS2* was associated to GAD in a sample of 607 adults exposed to 2004 Florida hurricane ($p = 0.026$) [48]. However, a recent study of 2661 individuals from the

Virginia Adult Twin Study of Psychiatric and Substance Use Disorders aiming to replicate the previous findings failed to find association to three most consistently associated SNPs from these previous studies [49]. Again these discrepant results may be due to differences in the phenotype definitions or ethnic background of the samples. However, twin studies suggest that many of these phenotypes share common risk factors [50], although it is not clear how strongly they are expected to relate to specific risk alleles and their effect size.

Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*Ppargc1a*)

Hettema et al. [51] combined data from several sources to identify and study 52 novel candidate genes for anxiety-spectrum disorders. They started with using strain distribution pattern analysis in heterogeneous stock mice that differ in anxiety-like behavior [29]. They then ranked these genes according to prior data including 1) extant linkage and knockout studies in mice, 2) a meta-analysis of human linkage scans, and 3) a preliminary human GWAS. Subsequently SNPs covering the nine top-ranked regions containing 14 genes were genotyped in a two-stage association study of subjects from the Virginia Adult Twin Study of Psychiatric and Substance Use Disorders chosen for high or low genetic loading for anxiety-spectrum phenotypes. Several SNPs within the transcriptional co-activator *PPARGC1A* associated with the anxiety phenotype. Initially *PPARGC1A* was discovered in the muscle cells and brown fat and characterized as a transcriptional co-activator, which stimulates mitochondrial biogenesis by increasing oxidative phosphorylation and by enhancing oxidative respiration [52]. Further studies indicated that *PPARGC1A* activates nuclear respiratory factor 1 (NRF1) and 2 (NRF2) [53]. These two genes are linked to oxidative stress, and involvement of oxidative stress in anxiety has been suggested by human and rodent studies, as discussed in recent reviews [54,55].

***Gabra2*, *Oprm1* and *TrkB* in PTSD**

Fear conditioning, a form of Pavlovian learning, has been used to model some aspects of PTSD. Parker et al. used an intercross of inbred mouse strains C57BL/6J x DBA/2J to identify, and an F8 advanced intercross line to fine-map, QTL associated with fear conditioning [56]. Subsequently, publicly available DNA sequence information and gene expression data were used to identify candidate genes based on the existence of non-synonymous coding polymorphisms and/or expression QTLs. Several candidate genes previously implicated in PTSD in humans were identified: gamma-aminobutyric acid receptor subunit alpha-2 (*Gabra2*), opioid receptor-mu1 (*Oprm1*), and neurotrophic tyrosine kinase (*TrkB*). *GABRA2* modulates

stress response [39] and SNPs within this gene have been associated with PTSD in adult patients previously exposed to child abuse [57]. OPRM1 has been linked with PTSD through different levels of μ -opioid receptor binding potential in a sample consisting of patients with PTSD (N = 16) and controls with (n = 14) or without (n = 15) combat exposure [58]. TRKB is a receptor for brain-derived neurotrophic factor (BDNF). Carriers of the Met allele of the *BDNF* Val66Met polymorphism show impaired fear extinction and disturbed fronto-amygdala activity [10]. In addition to these genes already linked to PTSD, Parker et al. found several other genes associating with fear conditioning in mice, and variants in the homologous human genes should be investigated as candidate genes for PTSD.

Gene expression profiling in brain tissue

Functional genomics experiments represent a data-driven approach for identifying associations between a phenotype and genes or gene networks. Based on the data, specific hypotheses can be formulated and tested *in vitro* and *in vivo*. Inbred mouse strains that differ in their innate anxiety levels have been used to identify gene expression patterns that correlate with behavioral phenotypes across a number of strains [59-61]. Fernandes et al. investigated gene expression in the hippocampus of eight inbred strains, which differ in many behavioral phenotypes, and identified 200 genes showing strain differences. The strongest genetic correlation with a phenotype was found for catechol-O-methyl transferase (*Comt*), a gene previously associated with aggressive behavior [59]. A panel of eight inbred strains was used by Letwin et al. to identify strain and brain region-specific expression differences in five brain regions. They identified several glutamatergic signaling pathway-related genes correlating with anxiety-like behavior [61]. We investigated gene expression differences in seven brain regions of six inbred mouse strains that differ in their innate anxiety levels [60]. We correlated gene expression patterns from seven brain regions, known to regulate some aspects of anxiety, with behavioral anxiety-measures and identified genes with an expression pattern that correlates with anxiety-like behavior. We then functionally verified by lentivirus-mediated gene transfer (overexpression and silencing by RNAi) that two genes, glyoxalase 1 (*Glo1*) and glutathione reductase (*Gsr*) regulate anxiety in mice [60]. Since *Glo1* has been identified by several studies using various approaches, it is discussed further in the next section. The challenge with the translation of the gene expression findings to human anxiety disorders is the poor availability of good quality post mortem brain samples. Another approach is to test if DNA variants in the homologous human genes confer predisposition to anxiety disorders, but since a large number of the gene expression changes are expected to be reactive rather than causal,

this approach may work better on a pathway than single gene level.

As a translational step we tested whether genetic variants in 13 genes shown to be differentially expressed between anxious and non-anxious mouse strains predispose humans to anxiety disorders. We carried out a genetic association analysis in a Finnish population-based Health 2000 Cohort consisting of 321 cases and 653 matched controls. Variants in six genes (*CDH2*, *ALAD*, *PSAP*, *EPB41L4A*, *DYNLL2*, and *PTGDS*) showed some evidence ($p < 0.01$) for association to anxiety disorders [62]. Interestingly, *Cdh2* was recently shown to confer susceptibility to compulsive behavior in dogs [63].

Glo1 has been identified through various approaches

Glo1 was one of the genes identified through gene expression profiling in inbred strains having a higher expression level in anxious strains [60]. In the same study, its overexpression in the cingulate cortex by lentivirus-mediated gene transfer resulted in increased anxiety-like behavior, while inhibition by overexpression of an shRNA decreased anxiety-like behavior. *Glo1* was independently identified through a genome-wide search for copy number variants (CNVs) in inbred strains [64]. It was shown that the difference in *Glo1* expression between inbred mouse strains is due to a CNV, the presence of which correlates positively with anxiety-like behavior. To show a causal relationship between the CNV and anxiety-like behavior Distler et al. generated BAC transgenic mice expressing different copy numbers of *Glo1* [65]. The mice with several copies have increased anxiety-like behavior, as expected. GLO1 is a detoxification enzyme, which together with glyoxalase 2 converts cytotoxic methylglyoxal (MG) to non-toxic form [66,67]. When exploring the molecular mechanism of GLO1 underlying anxiety behavior Distler et al. found that overexpression of *Glo1* reduces MG level in the brain. Moreover, they showed that MG is an agonist of GABAA receptors, and therefore reduced levels of MG decrease GABAA receptor activation [65]. This finding conforms well to the known involvement of GABAA receptors in the regulation of anxiety. Interestingly, two proteomics studies have also linked GLO1 with anxiety-like behavior. According to these studies GLO1 is down-regulated in the brain of two separate mouse strains selectively bred for high anxiety behavior compared to their respective low-anxiety strains [68,69], a finding contradictory to the findings in the inbred strains. This surprising difference is likely due to other alleles contributing to the anxiety phenotype in these models and other factors related to the selective breeding of the strains, including differences in initial allelic frequencies, linked alleles, and drift before or during inbreeding [70]. More detailed discussion on the role of GLO1 in behavioral phenotypes is found in an excellent recent review [70].

The role of *GLO1* in mental disorders has been studied in humans. Patients with major depression or bipolar disorder show reduced *GLO1* expression when in depressive state, but not during remission [71]. However, cholecystokinin-tetrapeptide (CCK-4), which is used to induce panic attacks, did not have an effect on *GLO1* mRNA levels in peripheral blood cells of 23 healthy volunteers [72]. In schizophrenia patients, rare genetic variants in *GLO1* have been associated with decreased enzyme activity and increased carbonyl stress [73]. Genetic association studies have been carried out in anxiety disorders. A common Ala111Glu substitution in *GLO1*, responsible for conformational change and decreased enzymatic activity, was investigated in 162 panic disorder patients and 288 matched controls from the Italian population [74]. Although there was no evidence of association to the overall diagnosis, some evidence was found for association with panic disorder without agoraphobia ($N = 61$ patients, $p = 0.015$). Similarly, Donner et al. failed to find strong evidence for association with this SNP and anxiety disorders in the Finnish population ($p = 0.021$) [62]. This functional SNP therefore does not seem to play a major role in the predisposition to anxiety disorders. Larger genetic studies are needed to find out whether other common or rare variants within *GLO1* are involved in the etiology of anxiety disorders.

Proteomic studies in mouse models

Altogether three proteomic studies have been carried out in bidirectionally bred mouse strains for high or low levels of anxiety. In the HAB/LAB mouse model several proteins have been identified, including *GLO1*, discussed already in detail above [69], and another interesting enzyme, enolase-phosphatase [75]. In a different bidirectional mouse model of anxiety-like behavior Szego et al. identified alterations in serotonin receptor-associated proteins [69]. Recent proteomic analysis of rat hippocampus after psychosocial stress revealed 21 differently expressed proteins. They were involved in various cellular functions, including signal transduction, synaptic plasticity, cytoskeleton remodeling and energy metabolism [76].

Since the proteomics-based methods are developing with fast pace, it is expected that they will in the near future reveal biomarker panels to be used in biological diagnostics of psychiatric disorders, in addition to shedding light to the neurobiological mechanisms regulating anxiety.

Conclusions

Because of their high prevalence, anxiety disorders impose high social and economic burden. Integration of data from several approaches is needed to understand the molecular mechanisms that regulate anxiety, and to develop novel pharmacological treatments. Genome-wide approaches to identify regulators of anxiety-like behavior in animal models

will greatly complement the ongoing GWAS efforts in human anxiety disorders. There are two major advantages in using mouse models compared to human patient samples. Since environmental factors can be controlled for, or specifically administered in animal models, the power to detect small genetic effects is likely better in animal models compared to human cohorts. Stress, especially in childhood, is a well-established risk factor for anxiety disorders, and several mouse models for childhood stress have been recently developed. These should be investigated in several inbred genetic backgrounds, to identify gene-environment interactions in controlled circumstances. Another benefit of using animal models is the ability to harvest brain tissue at any time point. This allows taking advantage of unbiased genome-wide and proteome-wide identification of genes that regulate anxiety. With mRNA-seq and small RNA-seq it is now possible to identify all expressed genes from a given tissue, at different time points. Bioinformatic integration of this information can then be used to identify dynamic gene regulatory networks, instead of single genes. Optogenetic manipulation of specific cell types, combined with behavioral and gene expression analysis will help to detect yet more specific circuits underlying anxiety behavior. This approach will require development of better methods to dissect specific cell types and to carry out RNA-seq from very small amounts of RNA.

Results from the animal models should be used to formulate and test specific hypotheses in humans, using genetic and imaging approaches. The progress of the translation has been hindered by the relatively small size of well-characterized anxiety disorder cohorts, as can be seen with examples given above. Also, anxiety disorders as a group are phenotypically heterogeneous and it is not expected that all genetic findings replicate across all phenotypes. Integration of results from human genetic and imaging approaches with mouse genetic and functional studies will be essential to understand the neurobiological basis of anxiety disorders, a prerequisite for targeted therapies.

Abbreviations

ALAD: δ -Aminolevulinatase; BAC: Bacterial artificial chromosome; BDNF: Brain-derived neurotrophic factor; fMRI: Functional magnetic resonance imaging; CCK-4: Cholecystokinin-tetrapeptide; CDH2: Cadherin-2; CNS: Central nervous system; CNV: Copy number variant; Comt: Catechol-O-methyl transferase; DYNLL2: Dynein light chain 2; EPB41L4A: Erythrocyte membrane protein band 4.1 like 4A; GABA: Gamma-aminobutyric acid; GABRA2: Gamma-aminobutyric acid receptor subunit alpha-2; GAD: Generalized anxiety disorder; GAD2: Glutamic acid decarboxylase 2; Glo1: Glyoxalase 1; Gsr: Glutathione reductase; GWAS: Genome-wide association study; HAB/LAB: High anxiety-like behavior/low anxiety-like behavior; HPA: Hypothalamic-pituitary-adrenal axis; MG: Methylglyoxal; NRF: Nuclear respiratory factor; OCD: Obsessive-compulsive disorder; Oprm1: Opioid receptor, mu 1; PPARGC1A: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PSAP: Prosaposin; PTGDS: Prostaglandin D2 synthase; PTSD: Posttraumatic stress disorders; QTL: Quantitative trait locus; Rgs2: Regulator of G-protein signaling 2; SNP: Single nucleotide polymorphism; TrkB: Neurotrophic tyrosine kinase.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

ES and IH contributed equally to this manuscript. Both authors read and approved the final manuscript.

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