Major anxiety-related interventions in rodent defense behaviors:

systematic review and meta-analyses

ABSTRACT

Assays measuring defense behavior assays in rodents, including the elevated plus maze, open field and light-dark box assays, have been widely used in preclinical models of anxiety to study the ability of therapeutic interventions to modulate the anxiety-like state. However, many important proposed anxiety-modulating factors, including genes, drugs and stressors have had paradoxical effects in different rodent studies. We performed a systematic review and meta-analysis of the literature on the effect of aimed to evaluate whether some supposed anxiety-targeted interventions actually had only trivial effects on rodent defense behaviors. Using PubMed search phrases to identify articles, a systematic review was conducted on a panel of anxiety-linked factors: 5-HT1A receptor, SERT, pain, restraint, social isolation, corticotropin-releasing hormone and Crhr1. Articles were included if they contained rodent defense behavior data for one of the anxiety interventions. Systematic meta-analyses were conducted for interventions with fifteen or fewer studies; randomly sampled meta-analyses of fifteen studies were conducted for interventions for which more than fifteen studies were available. Synthesis of the data was performed using random effects models of Hedges' *g*. Publication bias was found to affect several literatures. Surprisingly, three of the ten purported anxiety-related interventions had small and/or non-statistically significant effects. The literature supports seven of the ten factors as being strongly connected to anxiety-related behavior.

INTRODUCTION

The anxiety disorders are among the costliest classes of mental disorders, both in morbidity and economic cost (DiLuca and Olesen, 2014; Baldwin et al., 2014). Development of anxiety-reducing (anxiolytic) drugs has been a major focus of the pharmaceutical industry and academic neuropsychiatric research, though no new drug types have been adopted since the introduction of selective serotonin uptake inhibitors (SSRIs) and other antidepressants for the treatment of anxiety disorders (Tone, 2009; Griebel and Holmes, 2013). Anxiety research relies on similarities between human emotional behavior and behaviors in animals (Darwin, 1998), specifically rat and mouse (Prut and Belzung, 2003). While there are many rodent behavioral paradigms that aim to model anxiety-like behaviors, three related assays that specifically aim to measure anxiety-related defense behavior (ARDEB) have been established, also referred to as the approach-avoidance conflict tests (Griebel and Holmes, 2013). These assays are the elevated plus maze (EPM), the light-dark box (LD) and the open field (OF), the first, second and fifth most widely used assays in animal anxiety research, respectively (Griebel and Holmes, 2013). All three arena types used in these assays contain a sheltered domain (e.g., the closed arms in EPM) and an exposed region. It is thought that avoidance of the exposed portions of the chamber reports on anxiety-like states in the rodent brain. These assays are generally accepted as preclinical assays of anxiety disorders, by reference to classic studies that validated them with a panel of drugs known to have anxiety-modulating effects in humans (Crawley and Goodwin, 1980; Pellow et al., 1985; Simon et al., 1994).

Rodent research has been implicated in the largely frustrated efforts to develop new types of anxiolytics (Griebel and Holmes, 2013). The literature regarding defense behavior is contradictory about the size and even the direction of many interventions that are proposed to be anxiolytic or anxiogenic (together ‘anxiotropic’) (Prut and Belzung, 2003; Griebel and

Holmes, 2013). This is true even for some anxiety-related genes with major clinical relevance, such as Htt, the target of SSRIs. As with the assessment of clinical anxiety interventions (Baldwin et al., 2014), a solid evidence base of the effect of 'mainstream' anxiety interventions in the rodent ARDEB assays is necessary to guide decisions about further basic research and therapeutic development (Vesterinen et al., 2014). In light of the widespread discordance, we proposed the hypothesis that some of the purported anxiety-related factors had no substantial effect on ARDEB. We will refer to this as the "no-ARDEB-effect hypothesis." Secondary goals of this survey were to examine patterns in ARDEB evidence: gaps in the literature, heterogeneity and publication bias

To estimate the effect sizes of anxiotropic interventions, we first used an *ad hoc* literature search to identify ten claimed anxiety interventions and followed this with systematic reviews on all. From the articles identified, systematic or randomly sampled meta-analyses were performed, depending on the numbers of eligible articles available. The majority of purported anxiety-related interventions showed substantial effects on ARDEB. However, three supposed anxiotropic interventions showed only small and/or non-statistically significant effects on ARDEB. Our study also indicated that the Htt literature appears to be affected by publication bias.

METHODS

Literature review

Genes, drugs and environmental interventions that had been proposed to be involved in anxiety were identified by a literature search of review articles on the field of anxiety. Based on the history of anxiety research (Tone, 2009; Griebel and Holmes, 2013), a list of ten anxiotropic interventions were selected to be included in the systematic review, either due to their direct clinical importance (e.g., diazepam), their role as an example of a class of proposed anxiety-related factors (e.g., isolation), or their connection to a clinically important drug (e.g., Htt). A systematic review was conducted to identify published articles addressing experimental outcomes in rodents from the EPM, OF, or LD assays for these interventions. A comprehensive literature set for each genetic, pharmacological or environmental intervention was generated by a PubMed search using specific search phrases (Table 1). The selective serotonin reuptake inhibitors (SSRIs) will be the subject of a separate study currently in preparation, due to their clinical importance (Baldwin et al., 2014), the very large number of studies conducted on them (Griebel and Holmes, 2013), and their controversial efficacy (Kirsch et al., 2008).

Eligibility criteria and study selection

Bibliographic data (including PubMed ID, date of publication, title and abstract) of studies identified in the systematic review were exported to a spreadsheet using the PubMed export function. Each article on this list was then reviewed at four levels of detail (title, abstract, full text and a detailed review of experimental design) to determine their eligibility for the review. Studies were required to be written in English and to have reported ARDEB in adult rats or mice. We required that each included study contain (1) primary behavior data from either an OF, EPM, or LD experiment for the intervention of interest, (2) suitable controls were reported and (3) the relevant statistics were reported (mean, standard error or

standard deviation, and sample sizes of both control and intervention groups). Since the focus of this study was the direct effects of anxiety-related interventions on ARDEB, experiments that used combination treatments were excluded. For drug and environmental interventions, possible confounding effects of the role of developmental stage were reduced by excluding studies not performed in adult rodents. For gene knockout and overexpression interventions, only experiments using a lifetime loss of function were included in the meta-analyses.

Selection of non-anxiety-related interventions

Studies of the effect of non-anxiety-related interventions on ARDEB were harder to find, perhaps due to the bias against publishing negative results, and/or reduced likelihood of mentioning them in abstracts. PubMed searches in this area failed to identify any useful studies. Instead, a conventional *ad hoc* literature search was used to find reports of the effects of adenylyl cyclase 1 (two articles) and synaptotagmin (one article) on anxiety-related behaviors.

Data inclusion in meta-analyses

Of the ten systematic reviews, eight identified fewer than or equal to fifteen eligible studies and two identified more than fifteen (Table 1). For the first group, we used all available data in their respective systematic meta-analyses. As our aim was to survey the wider rodent ARDEB literature and not focus on any single manipulation, for interventions with more than fifteen eligible studies, a maximum of fifteen studies was randomly selected for inclusion. Sampling was done by randomly sorting the PubMed IDs in Excel and selecting the first fifteen articles in each re-ordered list. The included studies used the two-independent-groups design with sample sizes typically N = ~10 control animals and N = ~10 intervention animals. Thus randomly sampling fifteen studies would bring the total iterations to N = ~150, ~150. From the significance testing perspective, assuming modest heterogeneity (an

assumption that is false in a minority of cases) and a two-tailed t-test with alpha = 0.05 for a moderate (d = 0.5) effect size, such sample sizes would have power of ~99% (Faul et al., 2007). From the estimation perspective, precision would be predicted to improve by nearly 4-fold (= $\sqrt{150} \div \sqrt{10}$) relative to primary results, also assuming modest heterogeneity.

Data items and extraction

The following data were collected from each of the included studies: authors, year of publication, figure and panel numbers, species, genotype, and mean, standard error of the mean and sample size (N) of each intervention and its related control group. Graphically presented data were extracted from Portable Document Format (PDF) files with the Measuring Tool in Adobe Acrobat Pro. A sample of ten randomly selected experiments were re-extracted by a second reader to compare the effect of individual extractions; the difference between the two extractions was g = 0.03 [95% confidence interval (95CI) 0.01, 0.05] on average, a negligible influence on meta-analytic effect sizes.

Summary measures

The following behavioral metrics were extracted from the articles: in OF studies, percent or total time spent at the center; in EPM studies, percent or total time spent on the open arm; in LD studies, percent or total time spent in the bright area. To synthesize these time-based metrics from the three assays, all estimates were standardized to Hedges' g, where g is a preferred variant of Cohen's d that uses the pooled standard deviation and is corrected for bias using Hedges' method (Cumming, 2012; Borenstein et al., 2011). The conventional adjectives to describe effect size (trivial, small, moderate, large) are used where appropriate (Cumming, 2012).

Synthesis of results

Meta-analyses of experimental outcomes, including the calculation of weighted mean effect sizes (Hedges' *g*), 95% confidence intervals, I^2 heterogeneity values, and p values using the random effects model, were performed with the metafor package in R (http://CRAN.R-project.org/package=metafor) (Viechtbauer, 2010). All error bars in forest plots are 95% confidence intervals; forest plots were generated with metafor and custom R scripts and formatted in Adobe Illustrator.

Assessment of bias across studies

Publication bias was assessed using Egger's linear regression test for funnel plot asymmetry (Egger et al., 1997). The standard normal deviate (Hedges' *g* / standard error) for each study was regressed against the study's precision (1 / standard error) using the "lm" function in R (http://www.R-project.org/). For studies that had evidence of publication bias (p-value ≤ 0.05), the trim-and-fill method (Duval and Tweedie, 2000) was employed to estimate the effects of publication bias on the effect size estimate. Funnel plots and trim-and-fill adjustments were performed with the 'metafor' package in R (Viechtbauer, 2010).

RESULTS

Review selection criteria identified 179 eligible articles

The flow-chart in Figure 1 summarizes the study selection process. In total, 585 articles were identified by the initial PubMed searches. According to the selection criteria described above, 249 studies were excluded based on their titles and a further 65 were excluded based on their abstracts. The full text of the remaining 271 articles were screened for criteria related to experimental paradigm, methods, and relevant variables, resulting in the exclusion of a further 92 studies. A total of 179 articles were considered eligible for inclusion in the review. Random selection of a maximum of fifteen studies for each intervention reduced the final number to 87 articles for use in the meta-analyses.

Characteristics of included experiments

The characteristics of all included studies are given in Table 3. In brief, 87 studies comprising 97 EPM experiments, 37 OF experiments and 22 LD experiments were identified. Studies were published between 1993 and 2013 and included data from 109 experiments conducted on mice and 48 experiments on rats. ARDEB studies of diazepam used a median dosage of 1 mg/kg, with minimum and maximum dosages of 0.5 mg/kg and 5 mg/kg respectively. This dose range is similar to or higher than commonly used by patients.

Heterogeneity

Statistically significant heterogeneity was found in (8/12) of the meta-analyses. Only two meta-analyses had high heterogeneity (I^2 > 75%): Htr1a overexpression, and physical restraint (Higgins et al., 2003). Three of the meta-analyses, Htr1a knockouts and Htt knockouts and isolation, had moderate heterogeneity (50% < I^2 < 75%). However, the majority (seven meta-analyses) had low heterogeneity (I^2 < 50%). As most of these syntheses contained data from more than one assay type, it is encouraging that most had

low or moderate heterogeneity; this outcome is compatible with the idea that the three ARDEB assays are testing similar aspects of rodent behavior.

Publication bias

Censorship of statistically non-significant results can result in the overstatement of effect sizes in meta-analyses, for example this effect has a profound influence on the literature on rodent models of stroke (Sena et al., 2010). Publication bias in the ARDEB literature was assessed for the five meta-analyses that had at least ten experiments (Table 2) (Sterne et al., 2011). Funnel plots and Egger's linear regression test for asymmetry revealed that three of these literatures showed statistically significant bias (Figure 2; Table 2). Funnel plot asymmetry can arise from publication bias, the censorship of experiments showing small and/or statistically non-significant effect sizes (the file drawer effect) (Sterne et al., 2011). For the three biased data sets, we used trim-and-fill adjustment to correct for the funnel plot asymmetry and estimate the number of hypothesized missing studies (Duval and Tweedie, 2000). We conclude that the literatures of diazepam, Htt knockout and social isolation effects on ARDEB are affected by publication bias.

Diazepam produces a moderate reduction in defense behaviors

Diazepam is an important minor tranquilizer that was used for several decades as the first line of treatment for anxiety disorders (Tone, 2009) and, along with other benzodiazepines, is still used extensively to control anxiety (Baldwin et al., 2014). Recent clinical meta-analysis studies have found support for the efficacy of benzodiazepines in the short-term treatment of anxiety disorders (Baldwin et al., 2014). However, a published review of diazepam effects in open field studies revealed widespread disagreement between with 29 studies supporting an anxiolytic effect and 23 supporting either an anxiogenic effect or no effect (Prut and Belzung, 2003). We reviewed the available literature on diazepam for the three major rodent ARDEB assays: EPM, OF and LD. For the meta-analysis, data was

extracted from a randomized subset of fifteen studies (out of 62 eligible papers) (Brioni et al., 1994; Faria et al., 1997; Boerngen-Lacerda and Souza-Formigoni, 2000; LaBuda and Fuchs, 2001; Hui et al., 2002; Chen et al., 2004; Jain et al., 2005; Jászberényi et al., 2009; Frassetto et al., 2010; Wanasuntronwong et al., 2012; Onusic et al., 2002; Birkett et al., 2011; Costa et al., 2011; Choleris et al., 2001; González-Pardo et al., 2006). Calculation of an average Hedges' *g* (Cumming, 2012) revealed that diazepam had a moderate effect on ARDEB, with a -0.89 *g* [95CI -1.12, -0.6] reduction compared with untreated control animals (Figure 3). However, Egger's regression indicated the source literature was affected by publication bias, trim-and-fill adjustment reduced the effect size to -0.64 *g* [95CI -0.37, -0.91], a -0.25 *g* reduction in the anxiolytic effect of this drug. The meta-analysis had a low level of heterogeneity, indicating that the assay type, laboratory, dosage, species, strain and other possible sources of experimental variation played a relatively minor role.

5-HT1A receptor function influences ARDEB

Following negative publicity regarding the adverse effects of benzodiazepines (Tone, 2009), pharmaceutical companies focused on the serotonergic system (Griebel and Holmes, 2013). Of the fourteen mammalian serotonergic receptors, the serotonin receptor 5-HT1A has been targeted for its proposed connection with anxiety disorders and depression (Samuels et al., 2014). More than 1200 articles describe experiments relating 5-HT1A agonism to anxiety (Griebel and Holmes, 2013). However, a substantial proportion of those articles reported that 5-HT1A agonists or knockout of the Htr1a gene either produced no effect on anxiety or an effect that was opposite to the receptor's proposed mode of action (Griebel and Holmes, 2013). We performed systematic reviews of gene manipulations of Htr1a. Meta-analysis of data from eleven knockout articles (Ramboz et al., 1998; Gross et al., 2002; Groenink et al., 2003; Gleason et al., 2010; Freeman-Daniels et al., 2011; Ferrés-Coy et al., 2013; Klemenhagen et al., 2006; Parks et al., 1998; Vinkers et al., 2010; Zanettini et

al., 2010; Jain et al., 2012) revealed that removal of Htr1a produced a moderate increase (Hedges' g = 0.68 [95CI 0.38, 0.97] , p = 8.9 x 10^{-06}) in ARDEB phenotypes (Figure 4A). The three studies of Htr1a overexpression found by the review (Kusserow et al., 2004; Bert et al., 2006; Audero et al., 2013) indicate that this intervention moderately decreased ARDEB, but this was not statistically significant (g = -0.52 [95CI -1.26, 0.23], p = 0.18; Figure 4B). Overall, these results indicate there remains discordance regarding 5-HT1A function. While the knockout has a moderate anxiogenic effect, Htr1a overexpression does not have a statistically significant impact. A large cumulative sample size contributes to the ineffective overexpression result (N = 144). These results verify the no-ARDEB effect hypothesis for this treatment, indicating that Htr1a overexpression does not influence rodent defense behaviors.

Anxiotropic effects of the serotonin transporter

The serotonin transporter (SERT) is the target for the selective serotonin reuptake inhibitors (SSRIs), a class of drugs used to treat depression and anxiety (Baldwin et al., 2014). Meta-analysis of thirteen knockout studies (Holmes et al., 2003b; 2003a; Lira et al., 2003; Li et al., 2004; Carroll et al., 2007; Kalueff et al., 2007a; Olivier et al., 2008; Line et al., 2011; Schipper et al., 2011; Zhao et al., 2006; Kalueff et al., 2007b; Moya et al., 2011; Pang et al., 2011) revealed a large anxiogenic ARDEB effect (g = 0.88 [95CI -1.26, 0.23] , p = 5.2 x 10^{-14}; Figure 5A) produced by knocking out the SERT gene, Htt. However, a funnel plot and Egger's regression revealed a pronounced bias in reported effect sizes (Egger's test p = 6.7 X 10^{-6}, Table 2). Trim-and-fill adjustment filled the left segment of the funnel plot with ten imputed data points so as to obtain a symmetric funnel plot, reducing the effect size to g = 0.57 [95CI 0.29, 0.86].

Only two articles studying the effect of Htt overexpression on ARDEB were found (Jennings et al., 2006; Line et al., 2011). Meta-analysis revealed a large anxiolytic effect (g =

-0.94 [95CI -1.69, -0.20], p = 0.013; Figure 5B) in EPM and OF assays (no LD articles were found). The transporter gene knockout and overexpression effects clearly connect Htt function to ARDEB/ However, the direction of effects is the opposite of what would be expected from the clinical application of Htt inhibitors, given that SSRI reduction of Htt function is thought to have a therapeutic anxiety-reducing effect.

The effects of environmental stressors in ARDEB

Environmental stressors have physiological effects on animals that promote the anxiety-like state (van Praag, 2003). To survey a range of stress modalities we chose acute pain, bodily restraint and social isolation for review; all three are known to promote anxiety in humans (Sherif and Oreland, 1995). The systematic review identified only five papers measuring the effect of acute pain on ARDEB (Schellinck et al., 2003; Benbouzid et al., 2008; Parent et al., 2012; Leite-Almeida et al., 2012; Matsuzawa-Yanagida et al., 2008), but meta-analysis of these indicated a very large anxiogenic effect (g = 1.49 [95CI 1.12, 1.87], p = 5.8 x 10^{-15}; Figure 6A).

Review of fifteen studies of rodent bodily restraint (Carvajal et al., 2004; Lunga and Herbert, 2004; Estanislau and Morato, 2005; Hsu et al., 2007; Locchi et al., 2008; Nosek et al., 2008; Rylkova et al., 2009; Granjeiro et al., 2011; Reis et al., 2011; Anand et al., 2012; Walf and Frye, 2012; Busnardo et al., 2013; Liu et al., 2013; Ouagazzal et al., 2003; Chesworth et al., 2012) indicated that it had an overall moderate anxiogenic effect in EPM and OF assays (g = 0.70 [95% CI 0.82 - 1.32], p = 0.027; Figure 6B). The restraint meta-analysis indicated a high level of heterogeneity, I^2 = 87%; a sub-group analysis by assay type revealed that the different assays were not the source of this variability (data not shown).

Meta-analysis of fifteen articles (Rodgers and Cole, 1993; Haller et al., 2000; Blednov et al., 2001; Cheeta et al., 2001; Lapiz et al., 2001; Wright and Ingenito, 2001; Majercsik et al., 2003; Moragrega et al., 2003; Abramov et al., 2004; Wei et al., 2007; Koike et al., 2009; Santos et al., 2010; Cuenya et al., 2012; Lukkes et al., 2009; Hermes et al., 2011) on social isolation revealed it had a small anxiogenic effect (g = 0.37 [95CI 0.15, 0.58], p = 7.8 x 10^{-4}; Figure 6C). However, Egger's regression revealed funnel plot asymmetry, while trim-and-fill adjustment reduced the anxiogenic effect to only 0.15 g [95CI -0.10, 0.40], a small and statistically non-significant effect on ARDEB (Figure 2). It appears that, unlike the physical stressors, social isolation does not have an influence on ARDEB, thus supporting the no-ARDEB hypothesis.

The role of CRH and its receptor on ARDEB

Several neuropeptide-related genes involved in stress signaling have been linked to anxiety, notably the peptide, corticotropin-releasing hormone (CRH; also known as corticotropin-releasing factor) (Kormos and Gaszner, 2013) and its receptor, CRHR1. Two studies that examined the effects of Crh knockouts on ARDEB were found (Weninger et al., 1999), which revealed only a small effect (g = 0.30 [95CI -0.32, 0.92], p = 0.34; Figure 7A). This supports the idea that CRH does not influence ARDEB, and supports the no-ARDEB hypothesis. However, the meta-analytic result may suffer from insufficient power as the cumulative sample size was only 41.

CRH exerts its biological action via two receptors known as CRHR1 and CRHR2. The two receptors are pharmacologically distinct and only the former has been widely studied in the context of anxiety (Owens and Nemeroff, 1991; Paez-Pereda et al., 2011). Meta-analysis (Liebsch et al., 1995; 1999; Smith et al., 1998; Müller et al., 2003; Gammie and Stevenson, 2006; Trimble et al., 2007) found that, in contrast to the Crh knockout, deletion of Crhr1 had a large anxiolytic effect on ARDEB (g = -1.0 [95CI -1.30, -0.70], p =

6.64×10^{-11}; Figure 7B). The discordance between Crh and Crhr1 knockout effects has previously been attributed to the action of either urocortin or another unidentified peptide ligand of Crhr1 (Dunn and Swiergiel, 1999).

Genes with no purported effect on anxiety

Some of the interventions in the systematic review were found to have modest effects on ARDEB. We aimed to contextualize the magnitude of proposed anxiety factors with factors that the literature has not connected with anxiety. Such factors are not generally mentioned in anxiety reviews and it is difficult to identify them with PubMed searches as they are less likely to be published or, when published, are unlikely to be the focus of a study and thus may not be mentioned in the abstract. We adopted an *ad hoc* search for systems unimportant to anxiety, which identified adenylyl cyclase 1 (Storm et al., 1998; Krishnan et al., 2008) and synaptotagmin 1 (Powell et al., 2004) as being proposed to have no anxiety-related activity. Meta-analyses confirmed that both interventions have very small (Hedges' $g < 0.3$) and non-statistically significant effects on ARDEB (Figure 8A-B). These results indicate that anxiety factors generally appear to have an ARDEB effect that is at least twice the magnitude of factors believed to be unimportant to anxiety.

DISCUSSION

Summary of evidence

Inspection of the forest plots reveals that all of the primary publication sets include experimental effect sizes that are discordant, either in direction (anxiolytic versus anxiogenic) and/or magnitude. The generality of discordance in the literature emphasizes the critical utility of meta-analysis to behavioral neuroscience. Of ten analyses of putative anxiotropic interventions, seven yielded substantial meta-analytic effect sizes that were statistical and three produced non-statistically significant effect sizes, once publication bias was accounted for (Figure 9). Overall, these results verify the no-ARDEB-effect hypothesis in a minority of cases (Htr1a overexpression, Crh and social isolation): their consensus effect sizes are comparable with 'non-anxiety' genes like *Syt1*. However, the majority of putative anxiety-related genes do have at least moderate effects on the rodent defense behaviors. The synthetic data strongly confirm that diazepam, the serotonergic system, environmental stressors, and Crhr1 influence an anxiety-like process in the mouse brain.

Knowledge gaps, publication bias and heterogeneity

We found no knowledge gaps *per se,* as all the purported anxiety-related factors had at least two studies. Only Htt overexpression, Crh knockouts and the non-anxiety genes had total experimental iterations below N = 64, 64, the sample size needed for 80% power to detect a 0.5 standard deviation effect size. However, the Ht1a overexpression and CRH knockout meta-analytic results remain imprecise (Figure 9), indicating that more work could be done here. Of the five intensively published factors (15 studies or more), three were affected by publication bias, suggesting that publication bias affects a majority of literatures in this area. Heterogeneity was encouragingly low for the literatures of most of the factors reviewed, suggesting that laboratory, strain, assay type and protocol variations play a relatively minor role in these experiments.

Gains of function in Htr1a has a weak effect on ARDEB

Meta-analysis of Htr1a overexpression revealed that is has a moderate anxiolytic effect, but that this is not statistically significant. Substantial numbers of animals were used to obtain this results (N = 72, 72), though the meta-analysis also displayed a high level of heterogeneity (I^2 = 59%). That overexpression of Htr1a has a weak effect suggests that aiming to increase 5-HT1A function may be a poor strategy to reduce anxiety, despite the pronounced Htr1a knockout outcome. This conclusion is supported by recent clinical meta-analyses that have concluded that drugs targeting 5-HT1A - the azapirones - appear inferior to benzodiazepines for generalized anxiety disorder (Chessick et al., 2006) and that there is insufficient evidence to support azapirone use in panic disorder (Imai et al., 2014).

The Htt-SSRI paradox in defense behaviors

Drugs that target SERT, the SSRIs, are recommended as the first line of pharmacological treatment for anxiety (Baldwin et al., 2014). Blocking SERT-mediated transport (reuptake) of serotonin from the synaptic cleft is the proposed mechanism of anxiety reduction (Perez-Caballero et al., 2014). Rodent studies of chronic SSRI effects on ARDEB have been inconclusive (Perez-Caballero et al., 2014; Griebel and Holmes, 2013). Given the the inhibitors' clinical use, it is surprising that Htt knockouts have elevated anxiety relative to controls and that Htt overexpression reduces ARDEB phenotypes. The reason for this discordance is not clear. In some cases the authors of the primary Htt knockout studies have not discussed this paradox (Moya et al., 2011; Schipper et al., 2011; Carroll et al., 2007; Kalueff et al., 2007a). Other authors have remarked that the underlying reason remains unclear (Lira et al., 2003; Holmes et al., 2003b) or have questioned the validity of ARDEB assays (Pang et al., 2011). Primary study authors have also proposed two explanatory hypotheses. The first is that increased anxiety arises from developmental alterations present in Htt knockouts not present in chronically drug-treated animals (Olivier

et al., 2008; Zhao et al., 2006; Holmes et al., 2003b). This hypothesis could be tested with conditional knockdown models, i.e. in animals with Htt only deleted at the adult stage. While systematic review did not identify any published reports of post-developmental Htt knockout experiments (e.g., using floxed Htt), researchers have looked at the anxiety-related effects of conditionally ablating the Pet-1 gene. Pet-1 is a transcription factor with an expression pattern that overlaps closely with Htt's expression pattern. In mice with *Pet-1* removed in adulthood, mRNA levels of Htt are also reduced substantially (Liu et al., 2010). Like Htt knockouts, these mice show increased anxiety-like behaviors in multiple ARDEB assays (Liu et al., 2010), eroding confidence in the developmental alteration hypothesis. A second hypothesis to explain the Htt/SSRI paradox is that there is a J-shaped relationship between Htt function and anxiety, i.e., both wild-type and knock-out animals would have higher anxiety relative to animals with intermediate function (Olivier et al., 2008). Further study is required to fully understand the role of Htt and SSRIs in ARDEB.

Conclusions

This study supports the hypothesis that some mainstream anxiety-related interventions have trivial or unconfirmed effects in rodent defense behavior assays. However, this study also confirms a drug, two environmental stressors and a number of genes as authentic ARDEB influencers. These verified anxiety-related interventions (diazepam, Htr1a gene knockout, Htt gene knockout, Htt gene overexpression, acute pain, restraint and Crhr1 gene knockout) can be used as reference manipulations to validate other animal models of anxiety-like behavior.

Meta-analysis has the ability to aggregate information and resolve discordance in the primary literature. This ability may be particularly useful in behavioral neuroscience where most primary articles describe experiments with low power (or precision) (Button et al., 2013). Precise estimation of effect magnitude is important both to build quantitative

models of brain function and to improve the ability of preclinical studies to predict clinical efficacy.

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FIGURES AND TABLES

Figure 1. Flow chart of the systematic literature review of 10 anxiotropic interventions.

The literature was reviewed in a four-stage process, starting with a PubMed search that yielded 792 articles, followed by three screens of increasing detail, reviewing the article title, abstract, and full text for experimental design. A total of 91 articles were used in the meta-analysis. Further details are given in Table 1 and the Methods section.

Figure 2. Funnel plots of five meta-analyses.

Where at least ten experiments were available for meta-analysis, the effect sizes (Hedges' *g*) of the experiments are plotted against their respective standard errors. Points on each plot represent individual experiments. The triangle bounded by dotted lines indicates the area where 95% of studies are expected to fall, in the absence of both publication bias and study heterogeneity.

Figure 3. Meta-analysis of diazepam on rodent anxiety related behavior.

Meta-analysis of rodent diazepam effect sizes, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (N_C, N_T) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). The I^2 of 41% indicates a low level of heterogeneity.

Figure 4. Meta-analyses of serotonin receptor 1A interventions on rodent anxiety-related behaviors.

Meta-analysis of effect sizes of serotonin-targeted interventions is shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*.

The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (N_C, N_T) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: A. Serotonin receptor gene Htr1a knockout models. B. Htr1a overexpression.

Figure 5. Meta-analyses of serotonin transporter interventions on rodent anxiety-related behaviors.

Meta-analysis of effect sizes of serotonin-targeted interventions is shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (N_C, N_T) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: A. Serotonin transporter gene (Htt) knockout models B. Htt overexpression models.

Figure 6. Meta-analyses of experiments on the stress-anxiety relationship in rodents.

Meta-analysis of effect sizes of stress-anxiety interventions, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (N_C, N_T) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: A. Acute pain. B. Restraint stress (immobilization). C. Social isolation.

Figure 7. Meta-analyses of the effects of stress signaling genes on anxiety-related behaviors.

Meta-analysis of effect sizes of stress signaling genes, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (N_C, N_T) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: A. Crh gene knockout models. B. Crhr1 gene knockout models.

Figure 8. Meta-analyses of interventions known to be unrelated to anxiety.

Meta-analysis of effect sizes of non-anxiety-related interventions, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (N_C, N_T) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: A. Adcy1 gene knockout models. B. Synaptotagmin gene (*Syt1*) mutation models.

Figure 9. Summary effect sizes of all meta-analyses.

The weighted mean effect sizes of all 12 interventions are shown here. Each mean effect size is represented by the central vertices of a diamond; the outer vertices indicate the 95% confidence intervals. The horizontal axis is Hedges' *g,* the standard deviation change relative to control animals. Color indicates direction (green = anxiolytic, red = anxiogenic) and statistical significance (grey = statistically non-significant). The diamonds for the

diazepam, social isolation, and Htt KO meta-analyses represent the summary effect sizes after trim-and-fill bias correction.

Table 1. Summary of systematic reviews of anxiety-related interventions in mouse and rat.

The PubMed query phrases used to identify articles that might contain data relevant to the interventions and assays of interest are detailed. Title, abstract and full-text searches were performed to identify articles meeting the selected criteria. Where there were fewer than fifteen articles meeting the selection criteria, all relevant data in all articles found were used for data extraction and meta-analysis. Where there were more articles, we took a random sample of fifteen papers and used only relevant data therein for meta-analysis.

Table 2. Results of Egger's linear regression test for funnel plot asymmetry across six meta-analyses

Where at least ten experiments were available for meta-analysis, Egger's linear regression test for funnel plot asymmetry was performed. For each meta-analysis, the number of studies included, the vertical intercept of the linear regression, the corresponding 95% confidence interval for the intercept, and the p-value of Egger's test are listed.

Table 3. Characteristics of included experiments.

Characteristics of experiments from included studies are listed with Pubmed ID, year of study, and figure panel. The assay type, assay duration, variable used in experiment, route of injection, drug dosage, treatment duration, species, strain and gender are also detailed. Assay duration and treatment duration are listed in minutes. Dosage is listed in mg per kg body weight of animal. Cells containing NA indicate that the information is not available for the underlying intervention.

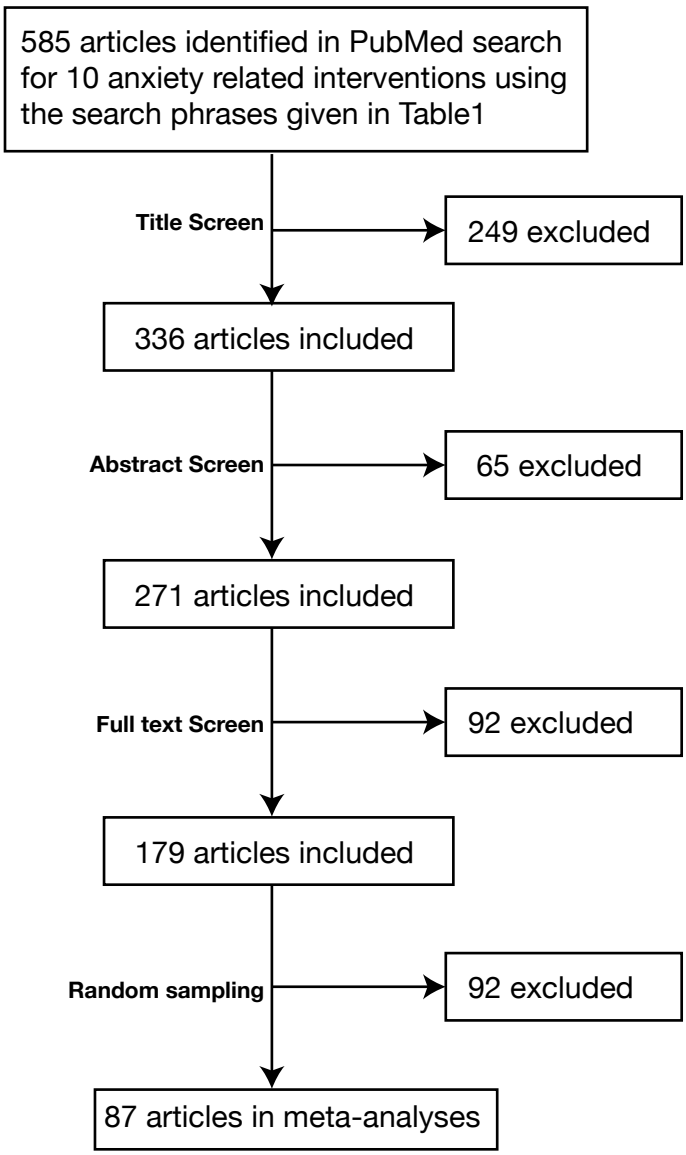


Figure 1. Flow chart of the systematic literature review of 10 anxiotropic interventions. The literature was reviewed in a four-stage process, starting with a PubMed search that yielded 585 articles, followed by three screens of increasing detail, reviewing the article title, abstract, and full text for experimental design. A total of 87 articles were used in the meta-analysis. Further details are given in Table 1 and the Methods section.

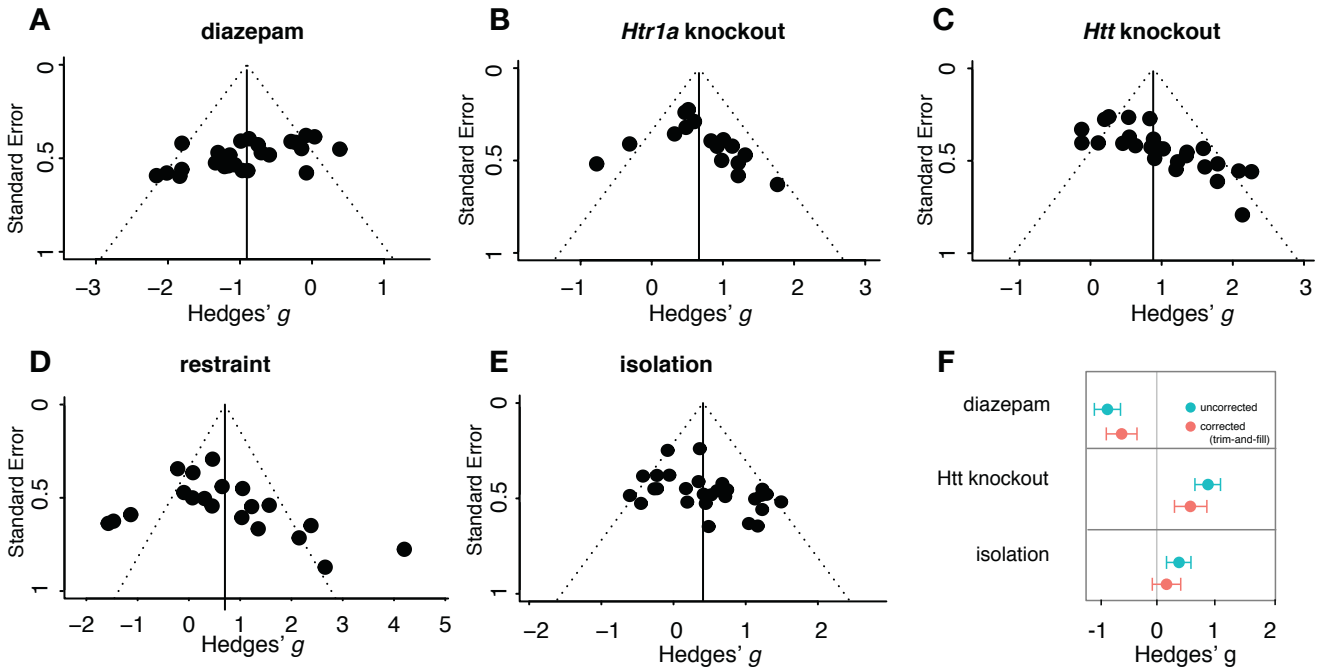
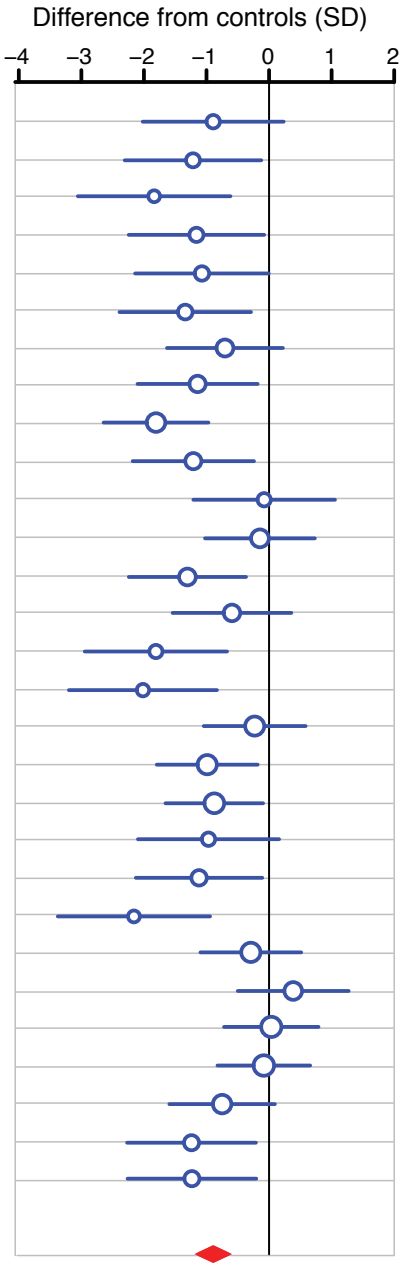


Figure 2: Funnel plots of six meta-analyses. A-E. Where at least fifteen studies were available for meta-analysis, the effect sizes (Hedges' *g*) of the experiments are plotted against their respective standard errors. Points on each plot represent individual experiments. The triangle bounded by dotted lines indicates the area where 95% of studies are expected to fall, in the absence of both publication bias and study heterogeneity. **F.** Trim and fill anlysis



Reference	Nc	Nt	Assay
Brioni 1994	8	6	EPM
Brioni 1994	8	8	EPM
Brioni 1994	8	8	EPM
Brioni 1994	8	8	EPM
Brioni 1994	8	8	EPM
Faria 1997	8	10	EPM
Boerngen−L 2000	12	8	EPM
LaBuda 2001	10	10	EPM
Hui 2002	16	16	EPM
Chen 2004	10	10	EPM
Jain 2005	6	6	EPM
Jaszberenyi 2009	10	10	EPM
Frassetto 2010	11	11	EPM
Wanasuntronwong 2012	9	9	EPM
Wanasuntronwong 2012	9	9	EPM
Onusic 2002	9	9	LD
Birkett 2011	14	10	LD
Birkett 2011	14	13	LD
Birkett 2011	14	14	LD
Costa 2011	7	7	LD
Wanasuntronwong 2012	9	9	LD
Wanasuntronwong 2012	9	9	LD
Choleris 2001	12	12	OF
Gonzalez−P 2006	10	10	OF
Birkett 2011	14	13	OF
Birkett 2011	14	14	OF
Birkett 2011	14	10	OF
Wanasuntronwong 2012	9	9	OF
Wanasuntronwong 2012	9	9	OF

diazepam $g = -0.89$, $p = 4.0 \times 10^{-14}$, $I^2 = 41$ %

Figure 3: Meta-analysis of diazepam on rodent anxiety related behavior. Meta-analysis of rodent diazepam effect sizes, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (NC, NT) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). The I^2 of 41% indicates a low level of heterogeneity.

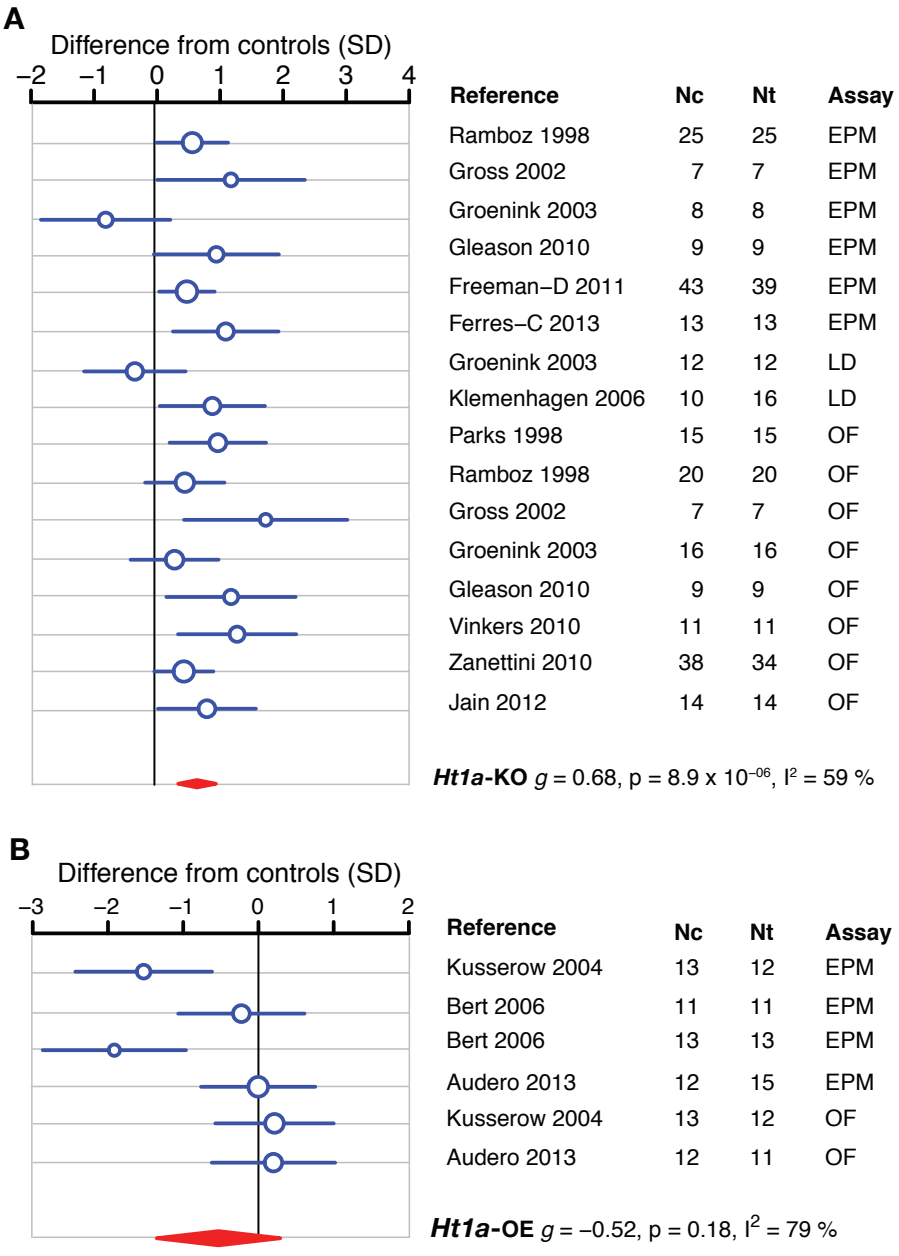


Figure 4: Meta-analyses of serotonin receptor 5-HT1A related genetic interventions on rodent anxiety-related behaviors. Meta-analysis of effect sizes of serotonin-targeted interventions is shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (NC, NT) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: **A.** Serotonin receptor gene Htr1a knockout models. **B.** Htr1a overexpression.

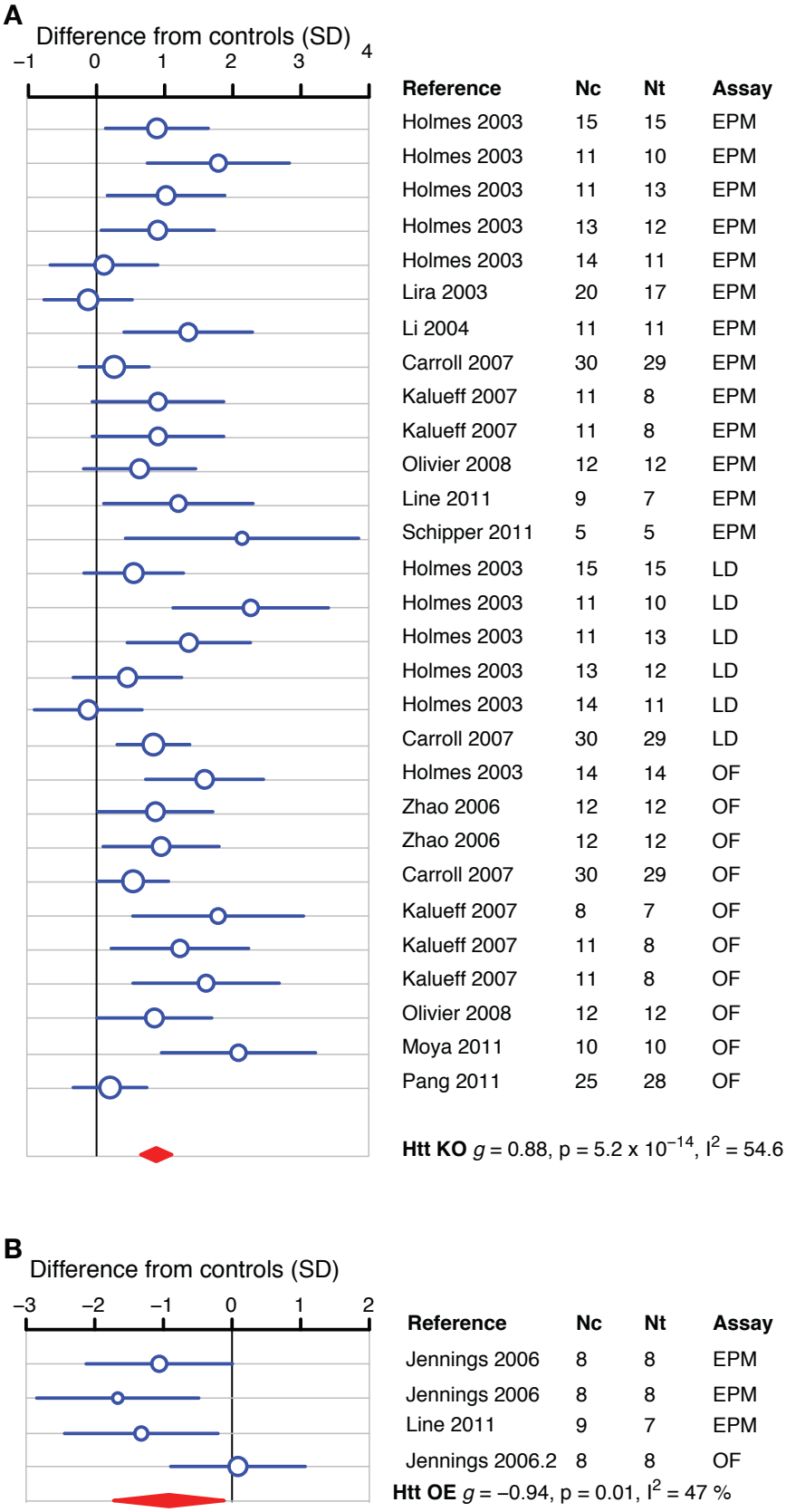


Figure 5: Meta-analyses of Serotonin transporter related genetic interventions on rodent anxiety-related behaviors. Meta-analysis of effect sizes of serotonin-targeted interventions is shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (NC, NT) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: **A.** Serotonin transporter gene (Htt) knockout models.. **B.** Htt overexpression models.

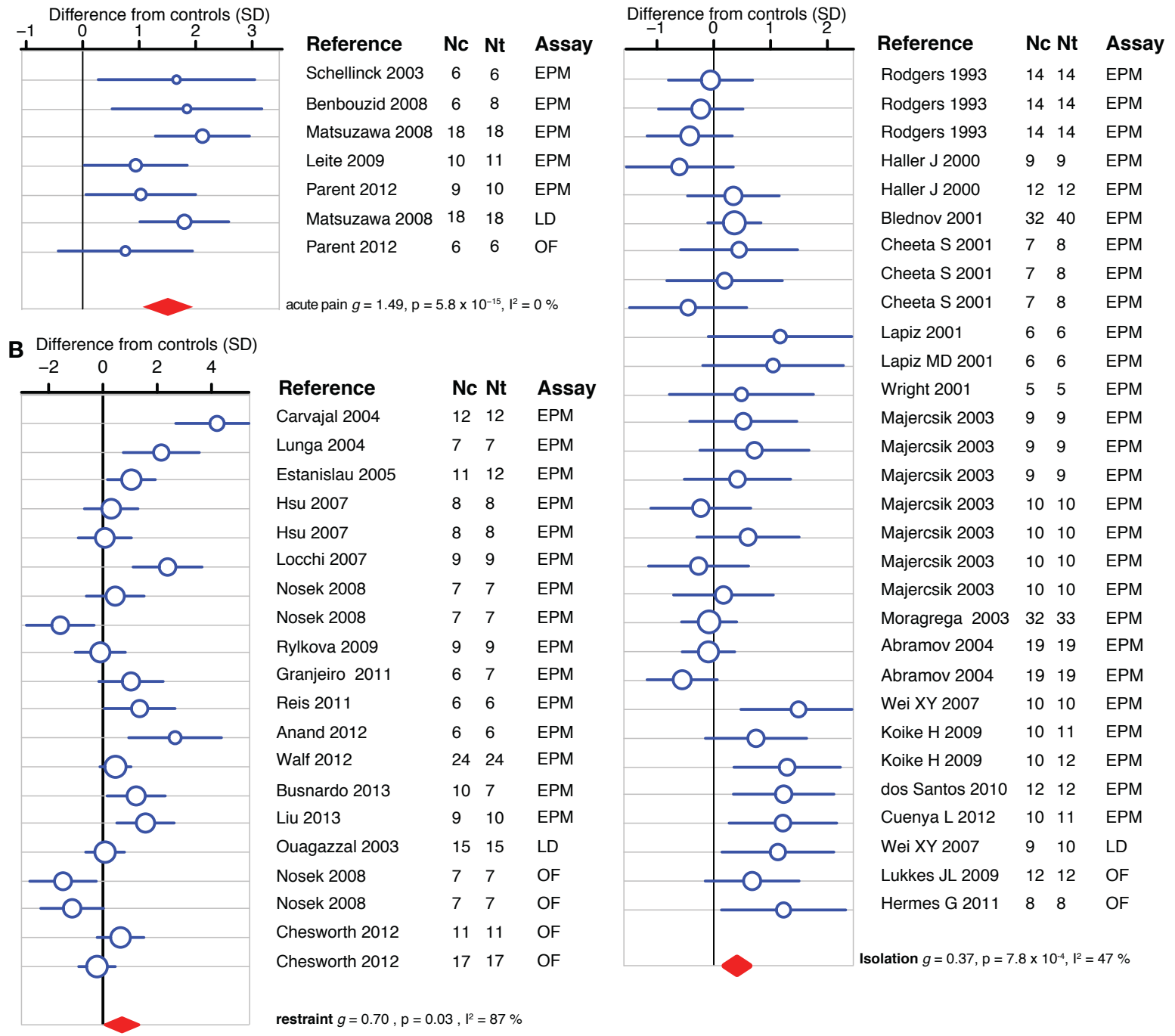


Figure 6: Meta-analyses of experiments on the stress-anxiety relationship in rodents. Meta-analysis of effect sizes of stress-anxiety interventions, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (NC, NT) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: **A.** Acute pain. **B.** Restraint stress (immobilization). **C.** Social isolation.

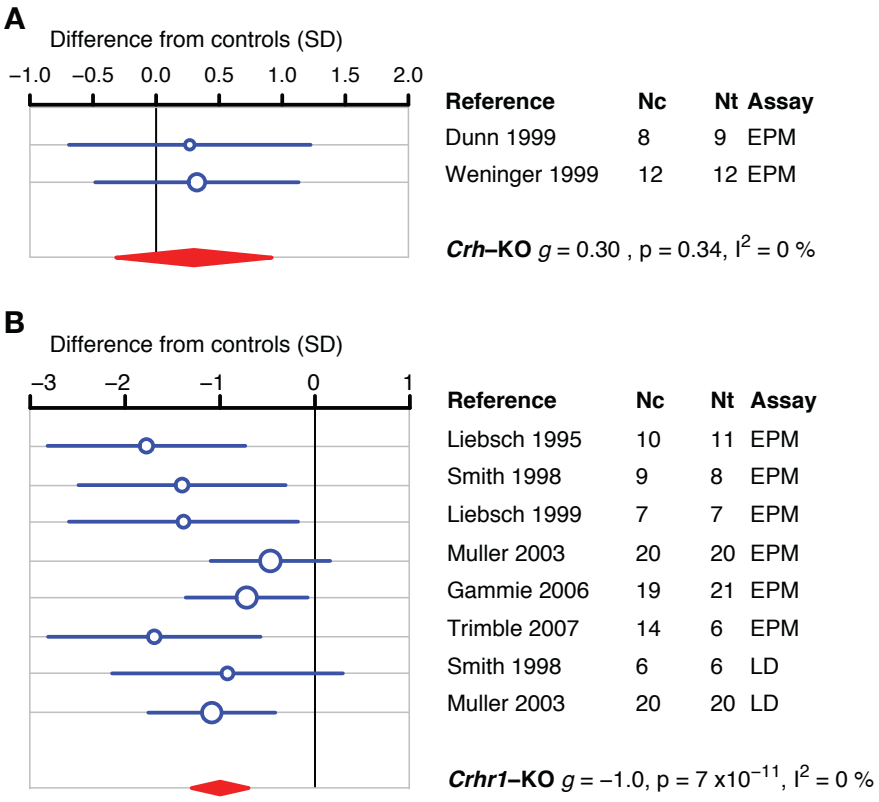


Figure 7: Meta-analyses of the effects of stress signaling genes on anxiety-related behaviors. Meta-analysis of effect sizes of stress signaling genes, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (NC, NT) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: **A**. Crh gene knockout models. **B**. Crhr1 gene knockout models.

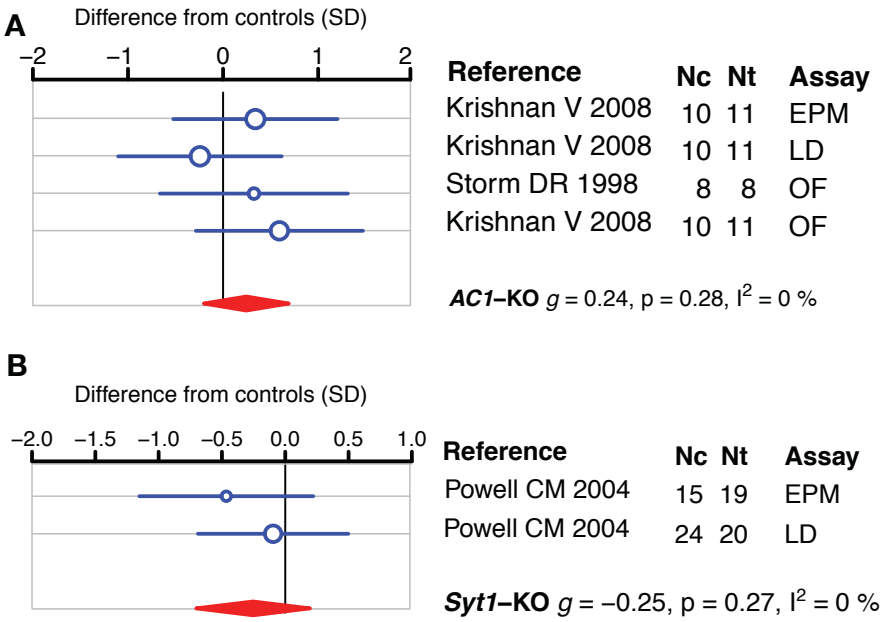


Figure 8: Interventions with no effect on anxiety-related behaviors. Meta-analysis of effect sizes of non-anxiety-related interventions, shown as a forest plot of standardized effect sizes (Hedges' *g*). Error bars indicate the 95% confidence intervals of *g*. The weighted average mean effect size of all studies is represented by the central vertices of a red diamond; the outer vertices indicate the 95% confidence intervals. Control and treatment samples sizes (NC, NT) and the assay types of the studies are given; elevated plus maze (EPM), open field (OF) and light-dark box (LD). Effects of: **A.** Adcy1 gene knockout models. **B.** Synaptotagmin gene (*Syt1*) mutation models.

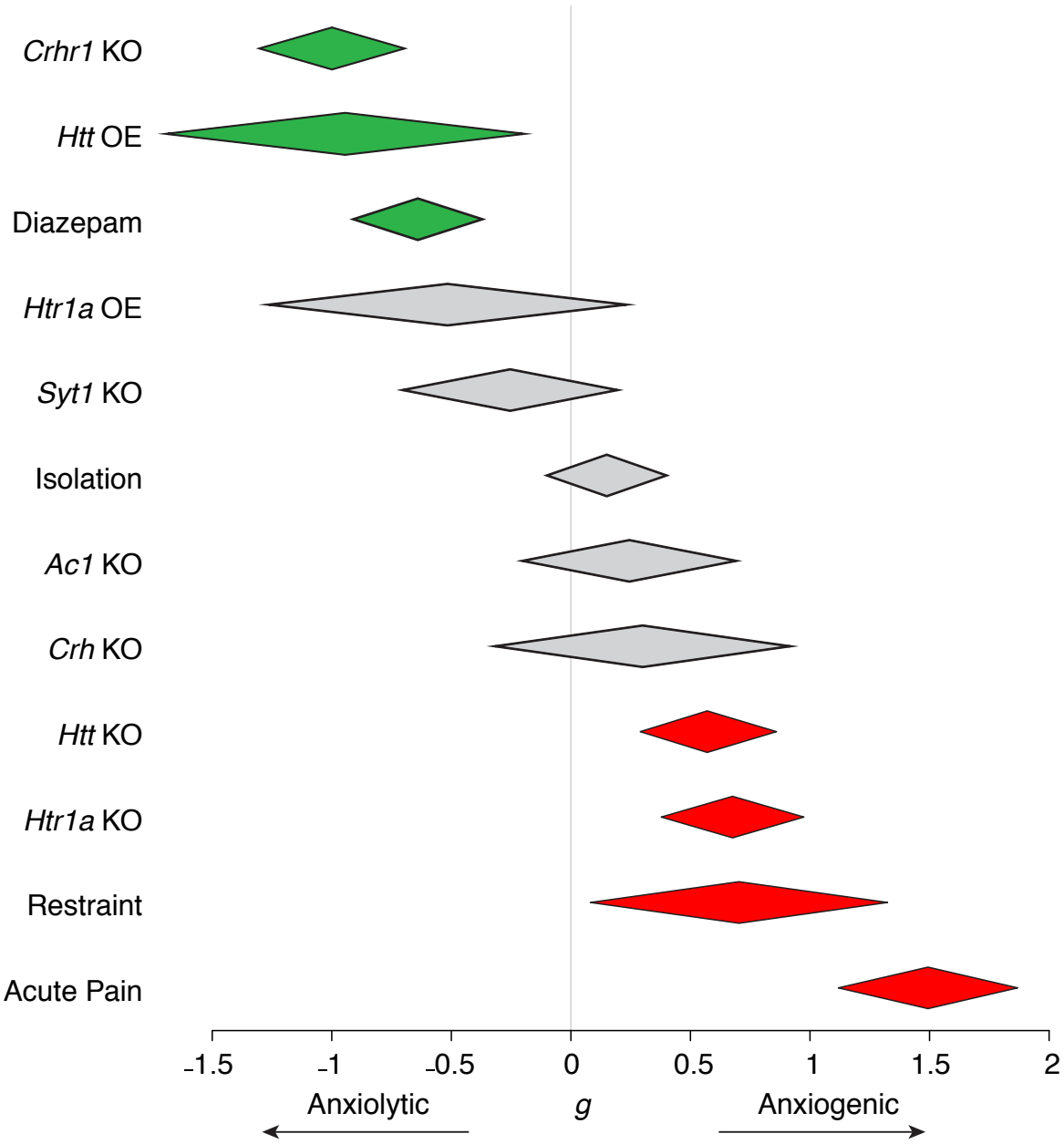


Figure 9. Summary effect sizes of all meta-analyses. The weighted mean effect sizes of all 12 interventions are shown here. Each mean effect size is represented by the central vertices of a diamond; the outer vertices indicate the 95% confidence intervals. The horizontal axis is Hedges' *g*, the standard deviation change relative to control animals. Color indicates direction (green = anxiolytic, red = anxiogenic) and statistical significance (grey = statistically non-significant). The diamonds for the diazepam, social isolation, and *Htt* KO meta-analyses represent the summary effect sizes after trim-and-fill bias correction.

Table 1. Summary of systematic reviews of anxiety-related interventions in mouse and rat. The PubMed query phrases used to identify articles that might contain data relevant to the interventions and assays of interest are detailed. Title, abstract and full-text searches were performed to identify articles meeting the selected criteria. Where there were fewer than ten articles meeting the selection criteria, all relevant data in all articles found were used for data extraction and meta-analysis. Where there were more articles, we took a random sample of ten papers and used only relevant data therein for meta-analysis.

Intervention	Query phrase used in PubMed for study selection	Articles identified by phrase	Articles meeting criteria	Articles in meta-analysis
Diazepam	(acute diazepam AND anxiety)) AND ((elevated plus maze OR open field OR light-dark) AND (rats OR rat OR mice OR mouse OR *Mus*)	96	61	15
***Ht1a* knockout**	(serotonin1A receptor OR 5-HT1A receptor AND knockout AND anxiety)	77	11	11
***Ht1a* over-expression**	(serotonin1A receptor) OR 5-HT1A receptor) AND (over-expression OR over-expression OR over-expressing) AND anxiety	13	3	3
***Htt* knockout**	(serotonin transporter knockout OR knockdown OR deletion OR antisense AND anxiety AND (elevated plus maze OR open field OR light-dark) AND (rats OR rat OR mice OR mouse OR *Mus*)	27	13	13
***Htt* over-expression**	(serotonin transporter AND anxiety AND (elevated plus maze OR open field OR light-dark) AND (increased OR over-expression OR overexpressing OR transgenic)) AND (rats OR rat OR mice OR mouse OR *Mus*)	13	2	2
***Crh* knockout**	(CRF OR CRH OR Corticotropin releasing factor AND knockout AND anxiety AND (elevated plus maze OR open field OR light-dark))) AND (rats OR rat OR mice OR mouse OR Mus)	34	2	2
***Crhr1* knockout**	(Corticotropin releasing factor receptor 1-deficient mice) OR CRH1 receptor antisense oligodeoxynucleotide OR Crhr1 null mutants OR Corticotropin-releasing hormone receptor antisense)	61	6	6
Pain	(Inflammatory pain AND anxiety OR neuropathic pain AND anxiety AND (elevated plus maze OR open field OR light-dark))) AND (rats OR rat OR mice OR mouse OR *Mus*)	42	5	5
Restraint	(acute restraint AND anxiety AND (elevated plus maze OR open field OR light-dark) AND (rats OR rat OR mice OR mouse OR *Mus*)	75	15	15
Isolation	(social Isolation OR single housing)) AND anxiety) AND (elevated plus maze OR plus-maze OR open field OR OFT OR light-dark)) AND (rats OR rat OR mice OR mouse OR *Mus*)	147	65	15
***Adcy1* knockout**	*Ad hoc* literature search	2	2	2
Synaptotagmin	*Ad hoc* literature search	1	1	1

		Egger's Linear Regression Test		Original (before trim-and-fill)		After trim-and-fill		
Meta-analysis	**No. of Experiments**	**Intercept [95% CI]**	**P-value**	**Hedges' *g* [95% CI]**	**P-value**	**Studies Added**	**Hedges' *g* [95% CI]**	**P-value**
Diazepam	29	-5.33 [-7.97 — -2.69]	**5.0×10^{-4}**	-0.89 [-1.12 — -0.66]	4.07×10^{-14}	8	-0.64 [-0.91 — -0.37]	2.56×10^{-6}
5HT1A KO	16	1.43 [-0.59 — 3.45]	0.19			N.A.		
Social Isolation	30	2.22 [0.45 — 3.99]	**0.02**	0.37 [0.15 — 0.58]	7.87×10^{-4}	7	0.15 [-0.10 — 0.40]	0.24
mSerT KO	29	3.83 [2.48 — 5.18]	**6.7×10^{-6}**	0.88 [0.65 — 1.10]	5.18×10^{-14}	10	0.57 [0.29 — 0.86]	9.71×10^{-5}
Restraint	20	2.93 [-0.34 — 6.19]	0.096			N.A.		

Table 2: Results of Egger's Linear Regression Test and Trim-and-fill for 5 Meta-Analyses

Shown here are the results of Egger's Test and the trim-and-fill operation for 5 meta-analyses that had ≥ 15 studies. For Egger's Test, p-values < 0.05 are shown in **bold**. For the trim-and-fill operation, the effect size for 3 meta-analyses are shown before and after trim-and-fill was performed. "N.A." indicates that the algorithm was not able to impute new studies to eliminate or reduce a non-negative linear intercept.