**The putative role of the relaxin-3/RXFP3 system in clinical depression and anxiety: a systematic literature review**

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**Abstract**

The relaxin-3/RXFP3 system is one of several neuropeptidergic systems putatively implicated in regulating the behavioural alterations that characterise clinical depression and anxiety, making it a potential target for clinical translation. Accordingly, this systematic review identified published reports on the role of relaxin-3/RXFP3 signalling in these neuropsychiatric disorders and their behavioural endophenotypes, evaluating evidence from animal and human studies to ascertain any relationship. We searched PubMed, EMBASE, PsycINFO and Google Scholar databases up to February 2021, finding 609 relevant records. After stringent screening, 51 of these studies were included in the final synthesis. There was considerable heterogeneity in study designs and some inconsistency across study outcomes. However, experimental evidence is consistent with an ability of relaxin-3/RXFP3 signalling to promote arousal and suppress depressive- and anxiety-like behaviour. Moreover, meta-analyses of six to eight articles investigating food intake revealed that acute RXFP3 activation had strong orexigenic effects in rats. This appraisal also identified the lack of high-quality clinical studies pertinent to the relaxin-3/RXFP3 system, a gap that future research should attempt to bridge.

**Keywords**

Relaxin-3; RXFP3; Neuropeptide; Depression; Anxiety; Neuropsychiatric disorder

**1. Introduction**

Given the continuously growing burden of clinical depression and anxiety (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018), considerable research has been conducted into elucidating the underlying mechanisms of these neuropsychiatric disorders. Yet, despite these efforts, the precise pathophysiology of mood disorders is not fully understood, although it is generally believed that they manifest as a result of the dynamic interplay between complex genetic and environmental factors (Hasler, 2010; Thibaut, 2017). Moreover, there is much heterogeneity in depression and anxiety (Lynch et al., 2020; Nandi et al., 2009) further complicating endeavours to understand their aetiology. Nonetheless, it has become increasingly evident that neuropeptides play an important role in modulating affective behaviour (Kormos and Gaszner, 2013; Thakker-Varia and Alder, 2009; van den Pol, 2012). As such, there is great interest in identifying and targeting relevant neuropeptidergic systems, which may then aid the development of novel therapeutic strategies.

In addition to several signalling systems, like those characterized by the neuropeptides oxytocin, neuropeptide Y, and galanin (Athira et al., 2020; Kormos and Gaszner, 2013), the relaxin-3/RXFP3 signalling system has emerged as a prominent example of promising neuropeptidergic circuits, having been the subject of considerable research since its discovery approximately two decades ago. Relaxin-3 is a neuropeptide predominantly expressed by GABAergic neurons in the nucleus incertus (NI) in rodents, though relaxin-3-expressing neuron populations have also been found in the pontine raphe nucleus, the periaqueductal gray and an area dorsal to the substantia nigra (Smith et al., 2010; Tanaka et al., 2005). The NI both receives inputs from and projects to various structures implicated in neuropsychiatric conditions, such as the hypothalamus, hippocampus, amygdala and prefrontal cortex (Goto et al., 2001; Olucha-Bordonau et al., 2003; Ryan et al., 2011); this overlaps with the distribution of RXFP3, the cognate receptor for relaxin-3 (Ma et al., 2007; Ma et al., 2009b; Sutton et al., 2004). Anatomical evidence thus supports the idea that relaxin-3 and RXFP3 together form a brain-wide arousal network, which regulates various behaviours linked to depression and anxiety, including stress responses, cognition, and appetite (Ganella et al., 2013b; Kumar et al., 2017; Olucha-Bordonau et al., 2018; Smith et al., 2014b). Studies employing animal models of behaviour provide further evidence of the function of this signalling system and these will be further discussed below.

While several review articles have provided an overview of the relaxin-3/RXFP3 system (Bathgate et al., 2013; Ganella et al., 2013b; Kumar et al., 2017; Ma et al., 2017b; Olucha-Bordonau et al., 2018; Patil et al., 2017; Smith et al., 2011; Smith et al., 2014b), none have done so using a systematic approach, which, according to Center for Reviews and Dissemination (CRD) guidance, involves stringent compliance with explicit, pre-specified and reproducible methodology (Tacconelli, 2010). In addition, none of these reviews has focused on mood and anxiety disorders exclusively, instead providing broad commentary on various domains of relaxin-3/RXFP3 signalling. We therefore conducted a rigorous systematic review of pertinent literature on relaxin-3/RXFP3 signalling in depression and anxiety, focused particularly on the behavioural manifestations of these psychopathological disorders. To thoroughly evaluate this putative relationship, both animal and human studies, with wide-ranging interventions, were included in this critical appraisal, culminating in a review of 46 animal studies (all with some behavioural component) and 5 observational clinical studies.

**2. Methods**

This systematic review was conducted in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standards (Moher et al., 2009) and Centre for Reviews and Dissemination (CRD) guidance (Tacconelli, 2010). Details of the protocols for this systematic review were registered on PROSPERO and can be accessed at <https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020207996> and <https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020204366>.

***2.1 Search strategy***

A literature search of English-language studies in the databases PubMed, EMBASE, and PyscINFO (via OVID), and Google Scholar, limited to the top 200 results sorted by relevance (Bramer et al., 2017; Haddaway et al., 2015), was conducted, with no limits on publication date. The initial search results were extracted in September 2020, and updated search results were extracted in February 2021 to ensure new studies published during the review process were included. Search terms comprised MeSH terms and keywords selected to identify studies that investigated the relaxin-3/RXFP3 system in the context of depression, anxiety and associated behavioural symptoms, which included appetite, sleep, and cognition. While these parameters can be altered even without the presence of depression or anxiety, it was important to include symptomology to capture the full, multifaceted scope of clinical depression and anxiety. For the full search strategy, see **Supplementary Table 1**. Reference lists of articles were also hand-searched to identify additional articles not found during the electronic database querying.

***2.2 Study selection***

All studies found by the search strategy were imported into EndNote software, after which duplicates were removed, initially with the automated duplicate-identification tool, and subsequently by manual examination of articles. All titles and abstracts were then screened against eligibility criteria; full-text papers of articles that passed initial screening were then obtained and scrutinised for potential inclusion in the review.

To comprehensively assess current evidence, both animal and human studies were included in the review. The following exclusion criteria were used to screen relevant studies: (1) publication is not from a peer-reviewed journal; (2) publication is a review, editorial, or conference abstract; (3) study does not include a control or comparator group; (4) intervention or exposure is not relevant or exclusive to the relaxin-3/RXFP3 system. Specifically for animal studies, records were further excluded when there was no *in vivo* component in the study design, or when there were no relevant behavioural outcomes reported. Specifically for human studies, records were further excluded when participants were under the age of 18, or when no well-recognised outcomes pertinent to depression, anxiety, or relevant symptoms were reported.

***2.3 Data extraction***

All data were extracted to corresponding pre-piloted fields in an Excel spreadsheet. Bibliographic information, including authorship, publication year, journal, and study identifiers, were extracted. In addition, details on study design, objectives, patient characteristics for human studies, and animal model characteristics for animal studies were noted. Descriptions of the relevant intervention or exposure were also recorded. When a study involved pharmacological or genetic interventions, various relevant details (dose, method of delivery, and timing for pharmacological interventions; method of genetic manipulation and genes studied for genetic interventions) were additionally extracted.

For animal studies, outcome data from various depression- or anxiety-like paradigms were extracted, including parameters for the forced-swim, large open field, elevated plus maze, and light-dark box tests. Furthermore, a wide range of anticipated behavioural outcome data were recorded, including parameters for food intake, alcohol consumption, Y-maze, social interaction test, and voluntary running wheel activity. When available, group averages and standard deviation or standard error of mean were obtained for these quantitative parameters; alternatively, test statistics were noted. When quantitative data was available only from graphs, plots were imported into Origin software, where the digitiser tool was then used to extrapolate data appropriately. Finally, any unanticipated but relevant outcome measures were extracted with a detailed text description.

For human studies, anticipated outcomes included recognised scales of depression and anxiety severity, binary presence or absence of these disorders, and a range of associated symptomology, such as metabolic and sleep disturbance. Detailed text descriptions of outcomes were extracted, in addition to appropriate group summary statistics or test statistics.

***2.4 Study quality and risk of bias assessment***

SYRCLE’s risk of bias tool (Hooijmans et al., 2014) was used to assess potential bias for all animal studies included in the review; this tool consists of 10 domains pertaining to selection, performance, detection, attrition, and reporting bias. In evaluating potential selection bias due to baseline characteristic differences (item #2), animal groups were considered acceptably comparable when sex and stage of development (comparable age ranges) were similar. In gauging potential detection bias due to lack of outcome assessor blinding (item #7), the use of automated behaviour assessment tools (such as automated tracking software) or objective quantitative measurements (such as weighing scale measures) were considered low risk. Consistency between the number of animals reported in the methods and results section was assumed to be sufficient evidence for a lack of attrition bias (item #8). Risk of bias information was summarised using bar charts and traffic light plots, created with dmetar and robvis packages in R (Harrer et al., 2019; McGuinness, 2019).

Initially, eligible human studies were to be assessed with the Cochrane risk of bias tool (Higgins et al., 2011). However, it became clear during the review that all relevant human studies in the literature were designed as cross-sectional or case-control studies. As such, case-control studies were instead evaluated using the Newcastle-Ottawa scale (NOS), which assesses selection, comparability, and exposure (Wells et al., 2013), while cross-sectional studies were evaluated with an adapted version of the NOS (Modesti et al., 2016), which assessed outcome instead of exposure. The assessment of selection and comparability domains concern study design and sampling, whereas assessment of exposure or outcome relates to the methodological rigor applied in measuring or analysing these variables.

***2.5 Data synthesis and analyses***

Study characteristics and results were summarised in tabulated form, and a narrative synthesis was constructed to explore any patterns and relationships between and within comparable studies. Meta-analysis (MA) was also performed for studies involving food intake, as it was the only outcome of interest common to an acceptable number of sufficiently homogenous studies. Using the meta package in R (Balduzzi et al., 2019), a random effects model was used to evaluate RXFP3 agonist effects on 1-hour and 2-hour food intake. Individual effects sizes were pooled to obtain standardised mean difference (SMD), particularly Hedge’s g statistic with its 95% confidence interval, which was chosen as it is most appropriate for the small sample sizes characteristic of most animal studies (Vesterinen et al., 2014). The *I*2, τ2, and χ2 statistics were obtained to assess heterogeneity between studies. A prediction interval was also calculated, as it takes into account study heterogeneity when presenting estimates of the true effects (IntHout et al., 2016).

**3. Results**

***3.1 Search and study selection***

Our electronic search of the literature identified 609 studies: 158 from PubMed, 201 from EMBASE, 50 from PyscINFO, and the top 200 relevant results from Google Scholar. Four additional articles were identified through reference list inspection. After removal of duplicates, 373 unique articles remained. Title and abstract screening excluded 293 articles, and subsequent full-text screening removed a further 29 articles, leaving 51 papers for inclusion in the review. **Figure 1** presents a detailed flowchart of this selection process.

***3.2 Animal studies with pharmacological intervention***

*3.2.1 Study Characteristics*

All animal studies involving pharmacological interventions are described and summarized in **Table 1**. Studies were conducted with rats, including Sprague-Dawley rats (n = 16), Wistar rats (n = 11), alcohol-preferring rats (n = 2), Fischer 344 rats (n = 1), and C57BL/6J mice (n = 5). A variety of pharmacological interventions, sometimes in tandem, were employed to explore the relaxin-3/RXFP3 signalling system; these include human or rodent relaxin-3 (n = 16), several selective RXFP3 agonists (n = 17), and several RXFP3 antagonists (n = 13). These compounds were administered through several routes, though the intracerebroventricular (icv) (n = 26) and intraparaventricular (n = 5) routes were most common.

*3.2.2 Animal models of depression and anxiety*

There were two studies that directly investigated depressive-like behaviour in rodents using the repeated forced-swim test (FST) model. The first study reported that icv administration of RXFP3-A2, a selective agonist for RXFP3 over RXFP1, decreased FST immobility time in male rats that were previously exposed to anxiety-like behavioural paradigms (Ryan et al., 2013a). Decreased immobility time is indicative of behavioural despair, suggesting that RXFP3-A2 had antidepressant-like effects in this subset of pre-tested rats. A more recent study corroborated these observations, finding that intranasally administered relaxin-3 (as well as a stapled RXFP3 agonist peptide) significantly decreased rat immobility and increased climbing behaviour during both test and retest sessions of the FST (Marwari et al., 2019).

Anxiety was assessed using several different paradigms: the light-dark box (LDB) test, the elevated zero (EZM) or plus maze (EPM) test, the large open field test (LOF), novelty-induced suppression of feeding test (NSFT), shock probe-burying test, and social interaction tests. In rat models, pharmacological activation of RXFP3 via icv or intranasal routes was associated with increased time spent in, and entries into, the light zone of LDB, indicative of decreased anxiety-like behaviour (Marwari et al., 2019; Ryan et al., 2013a). Similarly, icv or intranasal administration of relaxin-3 and RXFP3 agonists generally increased the percentage of time spent in, and entries into, open arms of the EPM/EZM (Marwari et al., 2019; Nakazawa et al., 2013; Ryan et al., 2013a). Furthermore, one study with male C57BL/6J mice found that acute icv RXFP3-A2 administration reduced FG-7142-induced anxiety-like behaviour in the EPM and LDB (Zhang et al., 2015), though notably, RXFP3-A2 did not affect basal EPM or LDB parameters. Intranasal administration of human relaxin-3 or a stapled peptide (14s21) was also associated with increased time spent in the centre and decreased latency to feed in the NSFT (Marwari et al., 2019), and icv relaxin-3 injection into male rats also decreased burying time in the shock probe-burying test (Nakazawa et al., 2013), further suggesting diminished anxiety-like behaviour in treated rats. In contrast to these findings, chronic RXFP3 activation in the ventral hippocampus (mediated by viral vector expression of R3/I5 agonist) induced anxiety-like behaviour in the LDB and EPM (Rytova et al., 2019), decreasing time spent in the light side and open arms, respectively.

RXFP3 activation, whether acute or chronic, exhibited no effect on LOF performance (Ryan et al., 2013a; Rytova et al., 2019; Zhang et al., 2015), except for one study that reported anxiolytic effects (increased time in the centre and reduced latency to the centre) upon intranasal relaxin-3 or agonist administration (Marwari et al., 2019). In addition, three studies used social interaction test paradigms to evaluate anxiety-like behaviour, with inconsistent results. Zhang et al. (2015) reported that icv RXFP3-A2 rescued FG-7142-induced decrease in social interaction during a single chamber social interaction test in male mice, while Albert-Gasco et al. (2019) reported that the same treatment in male rats had no effects on basal sociability in a three-chamber social interaction test. Yet, Rytova et al. (2019) demonstrated that chronic RXFP3 activation within the ventral hippocampus decreased social interaction, as reflected in diminished approach, follow, and sniff behaviours.

*3.2.3 Food and water intake*

Despite some heterogeneity between studies of food intake, eight studies with comparable designs were included in a MA of 1-hour food intake (de Avila et al., 2018; Hossain et al., 2013; Kristensson et al., 2015; Kuei et al., 2007; Lenglos et al., 2015; Marwari et al., 2019; McGowan et al., 2005; Shabanpoor et al., 2012), while six studies with similar designs were included in a MA of 2-hour food intake (Calvez et al., 2015; de Avila et al., 2018; DeChristopher et al., 2019; Haugaard-Kedström et al., 2011; Hida et al., 2006; Kristensson et al., 2015). These studies were adjudged appropriate for these analyses based on species, icv route of administration, outcome, and similarity of interventions (either relaxin-3 peptide or RXFP3 agonist administration). Across both MA, three of the included studies had multiple intervention groups that shared a single control group; since these interventions were of the same pharmacological nature (i.e., RXFP3 activation), they were combined into a single intervention group for each of the three studies. Furthermore, two studies in each MA stratified their findings (by gender, circadian rhythm, and agonist type), with independent control groups for each stratum; in these cases, each subgroup effect was treated as a single unit of analysis.

The meta-analyses, presented in **Figure 2**, indicated that pharmacological activation of RXFP3 via icv administration of exogenous peptides increased 1-hour food intake (SMD = 1.41 [1.06; 1.76], n = 10), as well as 2-hour food intake (SMD = 1.27 [0.91; 1.63], n = 8). There was moderate heterogeneity between studies included in the 1-hour food intake MA (*I*2 = 40%; τ2 = 0.0147; χ2 = 15.07, p = 0.09), though sensitivity analyses revealed that these metrics were skewed by a single study (Marwari et al., 2019) with a particularly large effect size. Nonetheless, the calculated prediction interval (0.91 to 1.91) suggests a clear 1-hour orexigenic effect for RXFP3 activation, even when accounting for study variability. In the 2-hour food intake MA, heterogeneity between included studies was low, with agreement between all diagnostic statistics (*I*2 = 0%; τ2 = 0; χ2 = 4.41, p = 0.73).

Two studies administering relaxin-3 via non-icv routes (see **Table 1** for details) also reported increased food intake at several timepoints (McGowan et al., 2006; McGowan et al., 2007). Moreover, there were four studies (Calvez et al., 2016a; Ganella et al., 2013a; Hida et al., 2006; Sutton et al., 2009) that investigated daily cumulative, rather than acute, food intake, all of which suggested an association between continued icv or intraparaventricular (iPVN) RXFP3 agonist administration and increased daily food intake. Smith et al. (2013b), however, demonstrated no change in food intake upon icv relaxin-3 or R3/I5 application, though this was studied in male C57BL/6J mice. There was also some inconsistency between the two food studies with RXFP3 antagonist application: while Smith et al. (2014a) reported reduced food consumption upon icv or iPVN R3(B1-22)R administration in male C57BL/6J mice, Ryan et al. (2013b) found no R3(B1-22)R effects on deprivation-stimulated food intake in male Wistar rats.

Two articles reported the effects of RXFP3 antagonism on food intake in the context of binge-eating. Kania et al. (2020) reported that iPVN administration of R3(B1-22)R reduced highly palatable food consumption in female rats that underwent frustration stress and caloric restriction to induce binge-eating behaviour; consistent with this, Calvez et al. (2016b) reported that central icv injection of R3(B1-22)R inhibited stress-induced increases of 1-hour sucrose intake in female binge-like eating prone rats. There were also nine studies that investigated water intake, with differing designs and results. Six studies reported increased water intake following injection of human or rat relaxin-3 via the icv route (Calvez et al., 2015; de Avila et al., 2018; Hossain et al., 2013; Marwari et al., 2019; McGowan et al., 2005; Otsubo et al., 2010). Among these studies, one also assessed the effects of RXFP3-A2 icv administration (de Avila et al., 2018) and another reported the effects of stapled peptide 14s21 icv administration (Marwari et al., 2019), which resulted in unchanged water intake and increased water intake respectively. One study reported no change in water following icv administration of R3/I5, RXFP3-A2 or an antagonist (Shabanpoor et al., 2012), while another two reported no change in water intake, upon food-deprivation stimulation or thirst challenges, following icv RXFP3 antagonist application (Ryan et al., 2013b; Smith et al., 2015).

*3.2.4 Relevant behavioural outcomes*

In addition to the above-mentioned outcomes, several other corollaries of behavioural symptoms linked to depression and anxiety were assessed, to ensure the scope of this review included an evaluation of the role of relaxin-3/RXFP3 in modulating relevant outcomes that characterize these neuropsychiatric disorders. For example, cognition was assessed with a spontaneous alternation task and three-chamber social test apparatus. Albert-Gasco et al. (2019) found that icv RXFP3 agonist-treated male rats showed no preference for novelty in the three-chamber test, indicating an inability to remember a familiar conspecific and thus social memory deficits. The same group also reported that RXFP3 activation impaired spatial working memory in the delayed spontaneous alternation T-maze task, with icv RXFP3-A2 treatment negating rat preference for a novel arm (Albert-Gascó et al., 2017). However, a different study found that icv R3(BΔ23-27)R/I5 (a RXFP3 antagonist) application also impaired spatial working memory, causing a reduction in percentage alternation score (Ma et al., 2009a). There were also two relevant studies of alcohol-seeking behaviour, in which administration of R3(B1-22)R centrally, into the bed nucleus of the stria terminalis, or into the central nucleus of the amygdala was shown to reduce alcohol self-administration and attenuate cue- and stress (yohimbine)-induced reinstatement of alcohol-seeking following extended extinction training (Ryan et al., 2013b; Walker et al., 2017).

General locomotor activity was predominantly unaffected after administration of either RXFP3 agonists or antagonists (Ganella et al., 2013a; Hida et al., 2006; Ryan et al., 2013a; Ryan et al., 2013b), except in a few instances where acute RXFP3 activation was reported to increase locomotor activity counts, distance, or duration (Marwari et al., 2019; Smith et al., 2013a; Sutton et al., 2009). In addition, several studies assessed locomotion as a secondary outcome during other testing paradigms, with a mix of increased (Albert-Gascó et al., 2017), decreased (Rytova et al., 2019) and unchanged (Albert-Gasco et al., 2019; Ma et al., 2009a; Zhang et al., 2015) locomotor activity following various treatments. In one instance, treating food-restricted mice with R3(B1-22)R reduced time spent climbing following food restriction, suggesting that RXFP3 antagonism reduced food anticipatory activity, though locomotor activity was again unaffected by the intervention (Smith et al., 2014a).

***3.3 Animal studies with genetic intervention***

*3.3.1 Study characteristics*

All animal studies with genetic interventions are described and summarised in **Table 2**. Once again, only rodent models were used, specifically Sprague-Dawley rats (n = 2), C57BL/6J mice (n = 10), and C57BL/6N mice (n = 2). While the most frequent genetic intervention was the use of relaxin-3 (n = 7) or RXFP3 (n = 4) knock-out mice (sometimes simultaneously), relaxin-3 knockdown with microRNA-499 into the NI (n = 2) and localized RXFP3 deletion via Cre-lox recombination (n = 2) were also employed. For more details of these studies and their respective interventions, see **Table 2**.

*3.3.2 Animal models of depression and anxiety*

Five studies used the FST to assess the effect of genetic interventions on depressive-like behaviour in rodents, with varying findings. Haidar et al. (2016) demonstrated that saline-treated female relaxin-3 and RXFP3 KO mice exhibited shorter latency to Porsolt posture than did saline-treated wild-type mice, which suggests greater depressive-like behaviour; this corroborates another report of an approximate 50% increase in rat immobility for male relaxin-3 KO mice, compared to wild-type mice (Smith et al., 2009). However, a later study from Smith et al. (2012) showed that male and female relaxin-3 KO mice undergoing a stress regimen did not display any differences in latency to Porsolt posture or immobility time relative to wild-type controls. Hosken et al. (2015) and Watanabe et al. (2011) made similar observations, reporting that RXFP3 KO and relaxin-3 KO mice, respectively, performed similarly to wild-type mice on the FST, with the latter study further highlighting the absence of genotype differences on the tail suspension test. Several of the aforementioned studies also employed the social interaction test: while two studies evidenced a lack of genotype differences in performance (Haidar et al., 2016; Watanabe et al., 2011), one report did find that relaxin-3 KO mice displayed less interactions with a novel conspecific, compared to wild-type mice (Smith et al., 2009).

The reduction of relaxin-3/RXFP3 signalling via genetic intervention, whether relaxin-3 KO, knockdown in the NI or conditional deletion in the dentate gyrus hilus, mostly did not alter performance on the LDB and EPM tests (Callander et al., 2012; de Ávila et al., 2020; Haidar et al., 2017; Shirahase et al., 2016; Smith et al., 2012; Smith et al., 2009). Hosken et al. (2015) did report that RXFP3 KO mice showed some signs of sex-specific decreased anxiety in these tests, with male KO mice making more entries into EPM open arms and female KO mice spending more time in the LDB light compartment, yet there were no genotype differences observed for all other parameters. Watanabe et al. (2011) and Haidar et al. (2016) too demonstrated that male relaxin-3 KO mice spend more time in (and in the former study make more entries into) open arms of the EPM, which suggests some level of decreased anxiety, but LDB performance was unaffected. Mice performance on the LOF test was also unaffected by whole-of-life relaxin-3 or RXFP3 KO (Haidar et al., 2019; Hosken et al., 2015; Smith et al., 2012; Smith et al., 2009; Watanabe et al., 2011), though relaxin-3 knockdown in the NI of female rats increased anxiety-like behaviour in the LOF test (de Ávila et al., 2020).

*3.3.3 Relevant behavioural outcomes*

In studies of whole-of-life relaxin-3 KO or localized relaxin-3 knockdown, there were generally no genotype or group differences in 24-hour food intake, nor in water or 1-hour highly palatable food intake (Callander et al., 2012; Hosken et al., 2013; Smith et al., 2013b; Smith et al., 2009). A recent report did highlight some contrasting findings, where miR499-mediated reduction of relaxin-3 in the NI of female rats produced a decrease in 24-hour food consumption at 4-weeks post-injection, though this change was not maintained at 5- and 6-weeks post-injection (de Ávila et al., 2020). The effects of relaxin-3/RXFP3 gene deletions on alcohol consumption were inconsistent. One group demonstrated that male RXFP3 KO mice and wild-type mice exhibited similar alcohol consumption and preference at baseline, though RXFP3 KO mice exhibited reduced alcohol preference following stress exposure (Walker et al., 2015a; Walker et al., 2015b). Another group reported increased alcohol intake and preference in male, but not female, relaxin-3 KO mice, relative to wild-type (Shirahase et al., 2016). The two groups also reported conflicting findings on sucrose consumption, with RXFP3 KO mice exhibiting attenuated sucrose self-administration and cue-induced reinstatement of sucrose seeking (Walker et al., 2015b), and relaxin-3 KO mice exhibiting no changes in sucrose consumption (Shirahase et al., 2016). There were no differences in saccharin consumption or preference between relaxin-3 KO, RXFP3 KO and wild-type genotypes (Haidar et al., 2016; Walker et al., 2015a).

Spatial working memory, assessed with plus or Y-maze spontaneous alternation tests, was largely unaffected by reduced localized or global relaxin-3/RXFP3 signalling (Callander et al., 2012; Hosken et al., 2015; Smith et al., 2012; Smith et al., 2009; Watanabe et al., 2011), although a single report of RXFP3 deletion from the dentate gyrus hilus demonstrated impaired spontaneous alternation and thus impaired spatial working memory in a Y-maze, as well as impaired spatial reference memory in the appetitive T-maze task (Haidar et al., 2017). The effects of RXFP3 deletion on the Morris water maze (MWM) task, which assesses spatial learning and long-term reference memory, was site-dependent: while RXFP3 deletion in the dentate gyrus hilus had no effect on MWM performance in one study (Haidar et al., 2017), RXFP3 deletion in the medial septum inhibited both MWM spatial search strategy adoption and long-term reference memory in another (Haidar et al., 2019). Compared to wild-type controls, relaxin-3 and RXFP3 KO mice exhibited no differences in recognition memory based on novel objection recognition performance (Hosken et al., 2015; Smith et al., 2012; Smith et al., 2009). In addition, a separate colony of male relaxin-3 KO mice did not exhibit any behavioural differences in the contextual and cued fear conditioning test, suggesting intact associative fear learning and memory (Watanabe et al., 2011).

Both relaxin-3 KO and RXFP3 KO C57BL/6J mice ran significantly less distance on voluntary running wheels during the active dark phase, which suggests that these KO mice displayed circadian hypoactivity (Hosken et al., 2013; Hosken et al., 2015; Smith et al., 2012). This difference disappeared when subjecting mice to prior food restriction, though relaxin-3 KO mice trended towards increased running wheel activity during the food anticipatory activity period (Hosken et al., 2013). Downregulation of RXFP3 activity, regardless of methodology and site, did not affect general locomotor activity (Callander et al., 2012; Haidar et al., 2017; Haidar et al., 2016; Haidar et al., 2019; Hosken et al., 2015; Smith et al., 2012; Watanabe et al., 2011), with the exception of a single study in which female relaxin-3 KO mice exhibited reduced baseline locomotor activity (Smith et al., 2009).

Prepulse inhibition of the acoustic startle response was also assessed in several studies, though it was mostly unaffected by genetic intervention, with relaxin-3 and RXFP3 KO mice displaying similar percentage prepulse inhibition as wild-type control mice, for both sexes (Hosken et al., 2015; Smith et al., 2012; Smith et al., 2009). However, Watanabe et al. (2011) demonstrated that male relaxin-3 KO mice exhibited an increased startle amplitude response and, conversely, a greater percentage prepulse inhibition compared to wild-type mice. This isolated finding differed in the protocol used for prepulse inhibition testing: the former studies delivered 70 individual prepulse-startle stimuli of four types, whereas the latter study presented mice with six blocks of six types of prepulse-startle stimuli (see Smith et al. (2012) and Watanabe et al. (2011) respectively for full details of the former and latter protocols).

***3.4 Human studies***

The comprehensive literature search yielded five human studies relevant to the relaxin-3/RXFP3 system, of which four were case-control studies and one was a cross-sectional, naturalistic observational study. Descriptions of the study designs and findings of these are presented in **Table 3.** Two of the case-control studies concerned metabolic phenotypes, with contrasting results. Ghattas et al. (2013) reported elevated plasma relaxin-3 levels in patients with metabolic syndrome, as well as significant correlations between plasma relaxin-3 and metabolic parameters, whereas, Zhang et al. (2013) found no such correlations in their cohort, nor any differences in relaxin-3 levels when comparing diabetes patients to controls. A more recent case-control study from Bangladesh reported decreased plasma relaxin-3 levels in patients suffering from major depressive disorder (MDD) and a negative correlation between relaxin-3 and depression severity, quantified by the Hamilton depression rating scale (Ali et al., 2020). Notably, the aforementioned assays used to measure plasma relaxin-3 levels were only briefly described and therefore could not be properly verified for accuracy. In addition, a post-mortem case-control study (Lee et al., 2016) showed parietal RXFP3 immunoreactivity was higher in depressed Alzheimer’s disease (AD) patients than in non-depressed AD patients and controls, though this study also reported no correlation between RXFP3 immunoreactivity and depression severity (which in this instance was assessed with a custom depression factor scale). It should be noted, however, that the antibodies used to assess RXFP3 immunoreactivity were not fully validated.

Finally, analysing a cohort of antipsychotic-treated patients, Munro et al. (2012) found associations between several single nucleotide polymorphisms of the relaxin-3/RXFP3 system and metabolic phenotypes: at RLN3, rs1982632 was associated with hyper-cholesterolemia; at RXFP3, rs42868 was associated with diabetes and hypercholesterolemia, while rs7702361 was associated with obesity and hypercholesterolemia; at RXFP4, rs11264422 was associated with body mass index (BMI).

***3.5 Study quality and risk of bias assessment***

*3.5.1 Animal studies*

The SYRCLE risk of bias assessment for animal studies is summarised in **Figure 3**, which highlights the proportion of studies at risk of a particular bias domain (unweighted because sample sizes in several animal studies were unclear). From this bar plot, it is evident that the quality of reporting for animal studies is fairly poor, with all studies lacking clarity for the allocation concealment, blinded interventions, and random outcome assessment domains. Moreover, over a quarter of studies were adjudged to have high risk of attrition bias, often due to ambiguous sample size reporting. A small number of studies were also deemed to have high risk of reporting and other miscellaneous sources of bias. For all other risk of bias items, over half the studies were at low risk, though a substantial proportion of studies were adjudged unclear. Traffic light plots indicating risk of bias for every individual study can be found in **Supplementary** **Figure 1**.

*3.5.2 Human studies*

The NOS for quality assessment of human studies is presented in **Table 4**. In general, the quality of case-control studies was subpar: three of the four included studies had shortcomings for case representativeness (item 2), control selection (item 3), control definition (item 4), exposure ascertainment and method consistency (items 7 and 8), and non-response rate (item 9). All three of these studies provided only crude descriptions of commercial radioimmuno- or enzyme-linked immunosorbent assay kits used in measuring serum relaxin-3 levels, hence their scoring deficiencies for the exposure ascertainment and method consistency items. The remaining case-control study explored RXFP3 immunoreactivity and was scored 6 out of a possible 9 stars on the NOS, indicative of adequate quality; however, this study too did not meet criteria for exposure ascertainment (item 7), as the antibody used in measuring RXFP3 immunoreactivity was not full validated. The single naturalistic observational study in the literature was also of reasonable quality, achieving 7 out of a possible 10 stars on the adapted NOS for cross-sectional studies, with only a description of non-respondents left completely unaddressed.

**4. Discussion**

***4.1 Summary***

Neuropeptides are thought to play a role in the aetiology of neuropsychiatric disorders, due to their modulatory and regulatory actions on neurotransmission. Indeed, research on this topic has identified several neuropeptides strongly implicated in depression and anxiety. For example, corticotropin-releasing factor (CRF) was discovered to regulate several stress-related behaviours in animal models, corresponding to its role in inducing activation of the hypothalamic–pituitary–adrenal axis; moreover, several clinical studies further point to CRF overactivity in depression, solidifying the involvement of this neuropeptide in the mood disorders (Arborelius et al., 1999; Waters et al., 2015). Substance P is another neuropeptide that has progressed our understanding of neuropsychiatric conditions, with animal studies suggesting a role in several behaviours, although clinical trials of antidepressants targeting this system have not, despite some initial optimism, been successful (Hafizi et al., 2007; McLean, 2005; Schwarz and Ackenheil, 2002). While research on the neuropeptide relaxin-3 and its cognate receptor RXFP3 is a relatively nascent field, if this neuropeptidergic system similarly modulates neuropsychiatric behaviours, understanding its function may provide insights into the molecular basis of mood and anxiety disorders, and could even propel the development of pharmacological treatments that target this signalling system.

Therefore, in this systematic review we aimed to elucidate the role of relaxin-3/RXFP3 signalling in depression, anxiety, and behavioural changes, such as appetite dysregulation, cognitive impairment, and circadian abnormalities, that constitute the associated endophenotype (Hasler et al., 2004). Despite some degree of inconsistency in the existing literature, largely due to the variability in study designs, this review identified a wealth of evidence demonstrating the manifold effects of relaxin-3/RXFP3 manipulation on various behaviours in animal models. These studies of animal behaviour are highly valuable and yield insightful findings, as they allow researchers to easily manipulate the relaxin-3/RXFP3 system with various interventions, a process that is significantly more constrained in clinical research. Such studies then theoretically provide rationale to translate findings from animals to humans. Our systematic search, however, identified only five clinical studies of the relaxin-3/RXFP3 system, all of which were observational in nature. Moreover, these human studies had several limitations, restricting their interpretation and value. This dearth of high-quality relaxin-3/RXFP3 research in humans further highlighted the patent need for more clinical studies of this understudied neuropeptidergic system.

The acute pharmacological activation of RXFP3 demonstrated antidepressant-like and anxiolytic effects in rat studies of fair methodological quality (adjudged accordingly as they did not obtain a high risk of bias judgement in any domain on the SYRCLE risk of bias tool, see **Supplementary Figure 1** for more details), particularly in the FST, EPM and LDB paradigms (Marwari et al., 2019; Nakazawa et al., 2013; Ryan et al., 2013a). That relaxin-3/RXFP3 signalling affects such behaviour is predicted based on the localisation of relaxin-3 and RXFP3 to pertinent anatomical structures in the rodent and primate brain (Bathgate et al., 2013; Ma et al., 2007; Ma et al., 2009b; Smith et al., 2010; Smith et al., 2014b). However, an important consideration is that in two of the aforementioned reports (Marwari et al., 2019; Nakazawa et al., 2013), rats were injected with native human relaxin-3 peptide, which can also bind to the receptor RXFP1 (Bathgate et al., 2013; Ganella et al., 2013b). Therefore, the notion that the anxiolytic and anti-depressant like effects reported are influenced by relaxin-3/RXFP1 interactions cannot be ruled out. Consequently, it would be of interest to explore how RXFP3 agonist administration, paired with the elimination of off-target actions on RXFP1 (by simultaneous administration of antagonists for example) would affect performance in the FST, EPM and LDB. Nonetheless, the results reported following administration of RXFP3-A2 (Ryan et al., 2013a), a selective agonist for RXFP3 over RXFP1, and the stapled peptide 14s21 (Marwari et al., 2019), a potent, high-affinity ligand for RXFP3, suggest that RXFP3 activation contributed, at least to some extent, to the observed modulation of depressive- and anxiety-like behaviour. There were also conflicting reports in rats and mice that RXFP3-A2 agonist administration did not affect basal FST (Ryan et al., 2013a) or LDB and EPM parameters (Zhang et al., 2015), but when these groups applied RXFP3-A2 to rats that were previously stressed, either by prior exposure to anxiety testing or, to mice, with FG-7142 induced anxiety, they observed reduced depressive- or anxiety-like behaviour in the same paradigms. This is in line with earlier reports that demonstrated increased relaxin-3 expression in rodent brains following exposure to restraint or swim stress (Banerjee et al., 2010; Lenglos et al., 2013; Tanaka et al., 2005); and together, these results suggest that the relaxin-3 network plays a key role in mediating behavioural alterations especially during responses to stressful conditions.

Some caveats must be considered, particularly regarding the findings on anxiety-like behaviour. Firstly, RXFP3 activation generally had no effect on LOF test performance; and although this may at first appear contrary to findings from the EPM and LDB, this is not unfeasible, as the LOF lacks an “escape” akin to the closed arms of the EPM or dark zone of the LDB. Additionally, a number of other known anxiolytic compounds have no effect on LOF performance, suggesting that it may not be the most effective test of anxiety (Prut and Belzung, 2003). Inter-test differences are unavoidable as all these paradigms are only models of features of anxiety in humans; therefore, future rodent studies of the relaxin-3/RXFP3 system should incorporate all three behavioural paradigms, as well as other tests such as the novelty-suppressed feeding test, when assessing anxiety-like behaviour, ensuring as complete an evaluation as possible. The other point of note pertains to a single study of chronic local RXFP3 activation within the ventral hippocampus, in which contradictory results were observed – increased anxiety-like behaviour and decreased social interaction following chronic R3/I5 expression and local secretion in the ventral hippocampus (Rytova et al., 2019). This could naturally be attributed to temporal differences between interventions, where transient and long-term RXFP3 activation may have opposing effects. Alternatively, the discrepancy between studies could be due to differences in the site of action; as the acute studies were done using icv or intranasal administration, resulting in broad RXFP3 activation, whereas the chronic study involved local secretion of R3/I5 into the ventral hippocampus. Given the specific and tightly-regulated contributions of hippocampal regions to anxiety-like behaviour (Bannerman et al., 2014; Jimenez et al., 2018; Revest et al., 2009), it is perhaps unsurprising that restricted, local RXFP3 activation had a distinctive effect. To resolve this apparent discrepancy, it would be of interest to study LDB/EPM performance and social interaction test response in cohorts of rats administered acutely with R3/I5 at different sites in the brain (including the ventral hippocampus), as well as another cohort subject to global, chronic R3/I5 secretion. This would allow comprehensive comparisons between studies to determine whether the observed behavioural differences are due to site-specific or temporal aspects of the different interventions.

Genetic interventions were also extensively employed to investigate the effects of relaxin-3/RXFP3 signalling on depressive- and anxiety-like behaviour in rodents; however, when evaluating these findings, some causes of inconsistency arise. At one level, there is the issue of result variability between the genetic studies that measured FST performance, where KO groups exhibited either increased depressive-like behaviour (in line with pharmacological findings) or no differences. These discordant findings may be due to differences in experimental design: one study with significant genotype differences was conducted and analysed predominantly in the context of methamphetamine treatment (Haidar et al., 2016), while a different null result study observed FST performance during a stress regimen (Smith et al., 2012). Moreover, both reports of significant genotype differences were adjudged to be at high risk of attrition bias, due to inadequate sample size reporting. As such, the notion of relaxin-3 KO mediated differences in FST performance must be approached with caution. In contrast to evidence from pharmacological studies, decreased relaxin-3/RXFP3 signalling via genetic intervention, whether KO, knockdown or Cre-lox deletion, generally had either extremely subtle or no impacts on tests of anxiety. Altogether, these results demonstrate a second level of inconsistency, between studies of genetic intervention and those of pharmacological interventions. There are several possible explanations for this disparity. Relaxin-3 and RXFP3 KO rodents may exhibit unaltered depressive- and anxiety-like behaviour due to the activation of compensatory mechanisms during life-long abolishment of relaxin-3/RXFP3 signalling. Compensation by similar pathways is arguably even probable given the pleiotropy and functional overlap of genes (Cryan and Mombereau, 2004), and several studies of KO rodents for depressive or anxious phenotypes that have previously proposed such phenomena (Bilkei-Gorzo et al., 2007; Edgar et al., 2011; Ramboz et al., 1998). KO studies also operate on a different timescale to the acute pharmacological studies (whole-of-life deletion vs transient activation), which may further contribute to the observed differences. With regard to relaxin-3 knockdown studies, we speculate that the injection of miR499-containing vector into the NI could have caused mechanical damage to the region; in turn, this may have disrupted other NI signalling pathways such that any potential effects of relaxin-3 silencing were nullified. Moreover, these knockdown studies were specific to the NI, while the pharmacological studies generally employed icv injection; as such, any inconsistency may be down to site-specific variation. Studies that employed Cre-lox deletion of RXFP3 were also targeted specifically to either dentate gyrus (Haidar et al., 2017) or medial septum (Haidar et al., 2019), meaning that contrasting results relative to pharmacological observations may again be explained by differences in site of action. This is further complicated by the possibility of targeting-vector diffusion to other regions in the brain, which may compromise efforts to deduce differential site-dependent relaxin-3/RXFP3 effects from these studies. Additional Cre-lox deletion studies targeting other brain regions would therefore be informative in determining site-specific actions of the relaxin-3/RXFP3 system.

While the systematic search retrieved no human studies of the relaxin-3/RXFP3 system in the context of anxiety, two studies relevant to depression were found. A case-control study from Bangladesh (Ali et al., 2020), which found decreased serum relaxin-3 levels in MDD patients, did not comprehensively outline their methodology and thus was only scored 3 out of a possible 9 stars on the NOS. As such, it is difficult to draw any solid conclusions from this report. On the other hand, a post-mortem case-control study that found increased RXFP3 immunoreactivity in AD patients with depression (Lee et al., 2016) was adjudged to be of reasonable quality on the NOS. However, this study somewhat lacks external validity, as it only examined depression in patients already suffering from AD, which could well have confounding effects. While these reports may initially seem contradictory, the RXFP3 upregulation observed in the latter study could feasibly be a compensatory response to the relaxin-3 ligand deficiency observed in the former study; this makes it difficult to interpret with certainty the direction of a possible underlying association between the relaxin-3/RXFP3 system and clinical depression.

A wealth of animal studies pertinent to food intake was identified, which is of interest since appetite change is a well-established component of the clinical depression endophenotype (Hasler et al., 2004; Simmons et al., 2016). Our meta-analyses identified a consistent increase in 1-hour and 2-hour food intake following acute RXFP3 activation with relaxin-3 or agonists in both Wistar and Sprague-Dawley rats, a finding that complements several reports of increased cumulative food intake during chronic RXFP3 activation. Here again it is important to note that, in the majority of studies used for meta-analyses, the native relaxin-3 peptide was administered, which might suggest that the orexigenic effects could be mediated by off-target actions on RXFP1. However, three studies additionally administered an RXFP3 antagonist prior to injecting either R3/I5 or RXFP3-A2 (Haugaard-Kedström et al., 2011; Kuei et al., 2007; Shabanpoor et al., 2012), demonstrating that the antagonist blocked the effects of these selective agonists, which when administered on their own increased food intake. In addition, chronic R3/I5 expression within the PVN increased daily food intake (Ganella et al., 2013a). Together, these observations highlight that RXFP3 activation is a major factor in the increased acute feeding ascertained in the meta-analyses. Moreover, while no similar paired-antagonist investigations were performed with native relaxin-3 peptide injection, icv relaxin-2 (the cognate ligand for RXFP1) administration in male rats has been shown to reduce food intake (McGowan et al., 2010), suggesting the orexigenic effects observed are related to RXFP3 rather than RXFP1 activation. Receptor selectivity is also particularly relevant to findings on water intake, which was largely increased following icv relaxin-3 administration. In this instance, it is likely that the dipsogenic effects of relaxin-3 are due to its activity at RXFP1, for several reasons. None of the studies that reported increased water intake following relaxin-3 administration demonstrated the blocking of such effects by an RXFP3 antagonist; in fact, the sole administration of RXFP3 antagonists had no effect on water intake. Furthermore, two investigations injected RXFP3-A2 into rats and observed no change in water intake, which suggests that RXFP3 activation alone does not mediate changes in drinking behaviour. The idea that relaxin-3 induces water intake via interactions with RXFP1 is further supported by previous studies that demonstrate dipsogenic effects of relaxin-2 (McGowan et al., 2005; McKinley and Johnson, 2004; Summerlee et al., 1998), as well as the presence of RXFP1 in the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT), which are circumventricular organs implicated in the control of body fluid homeostasis and consequently drinking behaviour (Bathgate et al., 2013; Gizowski and Bourque, 2018; Lind et al., 1984). It is thus feasible that icv injection of relaxin-3 would result in binding to RXFP1 at the SFO and OVLT, rather than RXFP3, to induce the increased water intake observed in rats.

In contrast to the meta-analyses of 1- and 2-hour food intake, relaxin-3 KO and knockdown in the NI did not affect food intake, with only one exception. The fact that increased RXFP3 activation had orexigenic activity does not necessarily imply that downregulation of relaxin-3/RXFP3 signalling should have anorexigenic activity. Indeed, while acute RXFP3 antagonist administration commonly reduced food intake, this finding was not absolute across pharmacological studies. Nonetheless, differences with the KO experiments may have manifest for the same reasons specified earlier. Moreover, it should be noted that KO experiments were conducted in C57BL/6 mice, while almost all pharmacological studies were conducted using rat strains. There are well-known behavioural differences between the two rodent species (Ellenbroek and Youn, 2016), which could then justify the apparent discrepancy. Moreover, the RLN3 gene is found on chromosome 19 in rats but on chromosome 8 in mice. This species-specific chromosomal localization of RLN3 could speculatively result in loss of synteny, which may explain the observed behavioural differences between the two species.

In addition to these animal investigations, several human studies explored a presumed link between the relaxin-3/RXFP3 system and metabolic alterations. Two case-control studies evaluated the relationship between plasma relaxin-3 levels and a panel of metabolic parameters, with one group finding weak but significant correlations, and the other finding none (Ghattas et al., 2013; Zhang et al., 2013). However, both studies obtained only 2 out of 9 stars on the NOS, with potential issues in all three domains of the scale; it is therefore difficult to properly interpret these opposing findings. An observational study of relaxin-3, RXFP3 and RXFP4 polymorphisms (Munro et al., 2012) fared much better in quality assessment, scoring 7 out of a possible 10 stars on the adapted NOS. Several single nucleotide polymorphisms across the relevant genes were found to be associated with metabolic syndrome phenotypes, including diabetes, obesity, and hypercholesterolemia, which may indirectly reflect appetite dysregulation. While the possibility of false positives among the reported associations cannot be ruled out, these observations seem to provide additional support to animal studies that suggest a putative role for relaxin-3/RXPF3 signalling in regulating appetite. Notably, however, this study has not since been validated, for several possible reasons. It may be that recruiting a large, sufficiently powered sample of participants comprising both metabolic syndrome cases and controls has been logistically difficult – even the 2012 study was specific to a cohort of antipsychotic-treated patients – or that other clinical studies of metabolic syndrome have overlooked the generally understudied relaxin-3/RXFP3 system. Alternatively, the lack of a follow-up study could be due to the increasing popularity of genome-wide association studies, stemming from continuous advancement in the field of genetics (Loos, 2020). Nonetheless, given the positive associations reported by Munro et al. (2012), a follow-up validation study of relaxin-3/RXFP3 polymorphisms and metabolic syndrome would be useful and informative.

Interestingly, genetic rodent studies consistently found that relaxin-3 and RXFP3 KO mice exhibited reduced voluntary running wheel activity during the dark phase (Hosken et al., 2013; Hosken et al., 2015; Smith et al., 2012), indicative of a circadian hypoactivity phenotype in these mice. Since sleep disturbance is a well-characterised symptom of clinical depression (Franzen and Buysse, 2008; Murphy and Peterson, 2015), this has noteworthy implications for the relaxin-3/RXFP3 system in affective disorders. Furthermore, evidence suggests that running wheel activity is a reward behaviour in rodents that reflects motivation (Mul, 2018; Novak et al., 2012), which then implies that relaxin-3/RXFP3 signalling may be key in modulating arousal behaviour and motivational drive. Such dysfunctional reward processing is another central characteristic of depressive disorders (Whitton et al., 2015) and running wheel activity has further been implicated in regulating the hypothalamic-pituitary-adrenal axis (de Rijke et al., 2005; Droste et al., 2007), which plays a major role in neuropsychiatric disorders (Faravelli et al., 2012; Varghese and Brown, 2001). Taken together, these running wheel studies suggest that relaxin-3/RXFP3 deficiencies may hamper reward processing and thus induce a depressive-like phenotype in rodents, which would complement findings from the acute pharmacologic RXFP3 activation studies. Studies of neuronal modulation at the NI, an area characterized by GABAergic projections that co-express relaxin-3 (Kumar et al., 2017; Olucha-Bordonau et al., 2018), provide additional support for relaxin-3/RXFP3 function in behavioural arousal: Lu et al. (2020) demonstrated that optogenetic activation of neurons at the NI, particularly projections to the medial septum and interpeduncular nucleus, promoted arousal (inferred from increased pupil diameter), locomotor speed, and theta power, while optogenetic inhibition of these neurons had the opposite effect. Furthermore, Ma et al. (2017a) reported that the chemogenetic activation of NI neurons promoted cortical electroencephalograph desynchronisation, which is associated with attention and wakefulness.

The relevant pharmacological studies of alcohol consumption further suggest that relaxin-3/RXFP3 plays a role in reward-processing, with icv and intra-bed nucleus of the stria terminalis RXFP3 antagonist administration decreasing baseline alcohol self-administration as well as reducing yohimbine-induced (a proxy for stress induction) reinstatement of alcohol-seeking (Ryan et al., 2013b; Walker et al., 2017). Two groups evaluated the effects of relaxin-3/RXFP3 genetic knockouts on alcohol consumption, with contrasting results, which is likely due to the specific whole-of-life knockout models employed. Shirahase et al. (2016) used relaxin-3 knockout mice, while Walker et al. (2015a; 2015b) used RXFP3 knockout mice; each particular gene knockout may trigger distinct developmental and compensatory mechanisms, culminating in the different alcohol-related behaviour observed. The differential sucrose consumption response between studies can also be attributed to the different gene knockout models; however, in this case, differing experimental designs between studies could underlie the observed inconsistency. More specifically, genotype differences were only found when an operant sucrose self-administration protocol was utilized (Walker et al., 2015b), as opposed to the use of a two-bottle choice test for sucrose or saccharin consumption (Shirahase et al., 2016; Walker et al., 2015a). Both these tests were designed to evaluate anhedonia in rodents, but both come with limitations. Performance on the two-bottle choice test can be affected by common laboratory practices that may act as stressors, such as tail handling or bottle switching, while sucrose self-administration can be influenced by rodent satiety and locomotor performance (Scheggi et al., 2018). More investigations that employ these rodent models of anhedonia, with strict controlling of possible confounders, would thus be valuable in resolving this apparent discrepancy.

Another outcome of interest was cognition, due to the relationship between cognitive deficits and neuropsychiatric disorders (Castaneda et al., 2008; Papazacharias and Nardini, 2012; Robinson et al., 2013). Social memory and recognition memory remained intact upon manipulation of the relaxin-3/RXFP3 system. However, findings for spatial working memory on spontaneous alternation tasks and long-term reference memory on MWM tests were extremely inconsistent within and between pharmacological and genetic studies. Nonetheless, several lines of evidence point to a role for the relaxin-3/RXFP3 system in modulating cognition. For example, hippocampal theta activity, which has been widely implicated in cognitive processes (Kahana, 2006; O'Keefe and Burgess, 1999; Solomon et al., 2019), was found to be enhanced following RXFP3 activation (Ma et al., 2009a). Moreover, several studies have highlighted how pharmacological and electrophysiological modulation of the NI can alter long-term potentiation (Farooq et al., 2013; Nategh et al., 2016; Rajkumar et al., 2016), while a recent study reported that the optogenetic manipulation of GABAergic neurons in the NI bi-directionally mediated memory formation in fear conditioning experiments: the activation of these neurons strongly suppressed freezing behaviour, indicating impaired contextual fear memory, while inhibition of these neurons resulted in the opposite behaviour, strengthening freezing behaviour (Szonyi et al., 2019). Since the NI is characterised by abundant relaxin-3 expression, this evidence suggests that relaxin-3/RXFP3 signalling and its possible interactions with other neurotransmitter systems have relevance for cognition, although the extent of this is unclear.

***4.2 Limitations***

Several of the included studies explored relevant behaviours with commonly used animal models, yielding interesting insights into the function of the relaxin-3/RXFP3 system. However, the interpretation of findings from these reports should be viewed with caution, as some of these animal models have inherent limitations worth considering. For instance, there has been much debate about the extent to which FST performance reflects depression in humans. Although its predictive validity is well-established, the test’s face and construct validity has been questioned because, while acute antidepressant treatment is effective in the FST, clinical application usually requires chronic treatment for any discernible effects, particularly for commonly used oral antidepressants like serotonin and noradrenaline reuptake inhibitors (Commons et al., 2017; Slattery and Cryan, 2012). However, the emergence of ketamine as a new treatment for depression may resolve this time frame discrepancy, as evidence suggests that ketamine can act as an effective and rapid-onset antidepressant (An et al., 2021; Corriger and Pickering, 2019). It has also been argued that the escape-motivated behaviour tested in the FST is driven by anxiety, such that the increased FST immobility time mediated by antidepressants reflect their anxiogenic effects rather than any antidepressant-like effects (Anyan and Amir, 2018). Nevertheless, this review integrated symptomatic aspects of depression in addition to the prevalent FST model, ensuring a more complete overview of the subject.

There were also constraints in assessing fully the risk of bias for animal studies, because a large proportion of studies did not mention certain domains, resulting in “unclear” risk of bias judgements; this is consistent with the generally poor quality of reporting pervasive in animal study literature (Kilkenny et al., 2009; Pound and Ritskes-Hoitinga, 2020). Furthermore, there was considerable heterogeneity in study design, interventions, and outcomes across the literature, which severely restricted possible meta-analyses; consequently, only the effect of acute RXFP3 activation on 1- and 2-hour food intake was quantitatively assessed. Even within these meta-analyses, the number of studies included in each was relatively low, such that conducting any sensitivity analyses with subgroups was not practical. Similarly, publication bias could not accurately be assessed with funnel plots (the standard in meta-analyses), as interpretation of publication bias from these plots is only reasonable when more than ten studies are included (Lau et al., 2006).

The limitations of clinical case-control studies identified in this review, reflected in the generally poor NOS scores, should also be carefully considered when interpreting reported findings. Three of these studies measured plasma levels of relaxin-3 as a key variable, but none performed any validation assays to corroborate selectivity, precision or robustness of the commercial radioimmunoassay or enzyme-linked immunosorbent assay kits used; instead, only brief mentions of the kit names were present in the articles’ methods section. The lack of information here severely constrains the evaluation of reported associations, as this leaves key concerns unaddressed. For example, it is not possible to determine whether the plasma levels of relaxin-3 truly reflect corresponding relaxin-3 levels in the brain, because relaxin-3 concentrations were not assessed in the brain or cerebrospinal fluid; all these investigations thus lack a key control. Though most highly expressed in the brain, relaxin-3 mRNA has been found in peripheral tissues such as the thymus, kidney and spleen (Bathgate et al., 2013; Bathgate et al., 2002), such that serum levels of relaxin-3 could feasibly be a composite of relaxin-3 from peripheral sources, which would further restrict the interpretation of these findings. The other post-mortem case-control study is also clouded by a major limitation, in that despite some attempts at characterization with immunoblot assays, the antibodies used to measure RXFP3 immunoreactivity have not been fully validated and characterized. Indeed, as Ma et al. (2017b) note, these commercial antibodies have not been tested in RXFP3 KO mice and so cannot be considered sufficiently specific. Another limitation of this post-mortem study is that agonal state of the post-mortem brain samples was not matched, such that some confounding is possible; however, it would be difficult to control for this factor given the limited availability of post-mortem tissue. Finally, in addition to limitations regarding the methods employed, it is worth noting that the sample sizes of these observational studies were relatively small, ranging from 43 to 419 samples. These studies are therefore unlikely to be of sufficient power to detect differences between case and control groups.

From a more general perspective, one may argue that the relatively small number of studies documented in this systematic review represents a possible weakness. The amount of research pertinent to the relaxin-3/RXFP3 system certainly does not compare to the number of investigations on other neurotransmitter and peptides, like the wealth of studies about serotonin in depression (Bacque-Cazenave et al., 2020; Cowen and Browning, 2015); this gap makes sense given the comparative infancy of the relaxin-3/RXFP3 field. However, the number of studies identified is not a controllable factor, but rather one of several insights generated while executing an exhaustive, pre-defined search strategy free of bias (as per the protocols registered on PROPSERO for this systematic review). Furthermore, the scarcity of studies is in fact a key finding of this review, in that the small but interesting set of articles identified underscore the need for further exploration of the trends observed, albeit with better methodological reporting and rigor, especially in future clinical investigations.

***4.3 Future directions***

Since current pharmacological studies on this topic have only assessed normal rodent performance in behavioural paradigms, it will be of interest to investigate the relaxin-3/RXFP3 system in other rodent models that exhibit elevated anxiety- or depressive-like behaviour. For instance, the Wistar-Kyoto strain of rats exhibit several traits reminiscent of depression, such as an exaggerated stress response and hypothalamic–pituitary–adrenal axis dysregulation (Will et al., 2003), in addition to increased immobility in the FST. Other examples are the Roman Low Avoidance (Escorihuela et al., 1999; Steimer and Driscoll, 2005) and Syracuse Low Avoidance rats (Brush, 2003; Brush et al., 1999), both of which are strains that have been selectively bred to exhibit higher anxiety-like behaviour on the traditional anxiety paradigms. In addition, assessing depressive-like behaviour with a repertoire of tests to more succinctly model the depression endophenotype (for example, more frequently integrating the sucrose preference or operant self-administration test for anhedonia) would help circumvent possible criticism of the FST in future studies. Thus, the pharmacological manipulation of relaxin-3/RXFP3 signalling in psycho-genetically selected rodents, combined with a more comprehensive approach to modelling depressive-like behaviour, may provide further understanding of how RXFP3 modulation affects behaviour in more relevant animal models of depression and anxiety. In addition, there is a need to elucidate the different site-specific effects of relaxin-3 or RXFP3 agonist administration; studies of chronic RXFP3 activation via the icv and iPVN routes are certainly warranted, while investigating acute RXFP3 activation via local injections at relevant sites may provide interesting comparative insights.

In addition to questions over site-specific effects of relaxin-3/RXFP3 signalling, existing animal studies also reveal that there is a lack of understanding of the precise temporal control of relaxin-3/RXFP3 signalling. The advent of optogenetics and chemogenetics has enabled remarkably specific control of neuronal activity (Galvan et al., 2018; Kim et al., 2017), and future research could therefore take advantage of these technologies to finely control activation or suppression of relaxin-3/GABAergic neurons in different regions of interest, which may enable a conclusive evaluation of how changing timescales and varying sites of action may mediate differential behavioural changes. Novel findings could then help resolve the discrepancy that presently exists between pharmacological and genetic studies. It should be emphasized however that future animal research of the relaxin-3/RXFP3 system must more stringently adhere to reporting standards for animal research, such as the ARRIVE guidelines (Percie du Sert et al., 2020), as the existing literature is far too ambiguous in their methodological reporting, reflected in the large proportion of “unclear” judgements on every domain of the SYRCLE risk of bias tool (see **Figure 3**).

This review also underscored a significant gap in the literature, namely the distinct lack of human studies relevant to the relaxin-3/RXPF3 system, particularly studies of satisfactory quality. Before proposing potential future studies, it is important to stress that future clinical studies on this topic must be more rigorous in their methodological approach and reporting standards. Assuming these criteria are met, one would ideally track real-time relaxin-3/RXFP3 signalling activity in live human brains to evaluate its role in mediating behaviour; unfortunately, there is not yet a validated method to assay this. There are also several other possible avenues to explore. For example, follow-up clinical studies investigating relaxin-3/RXFP3 genetic polymorphisms or relaxin-3 serum levels in depression and anxiety are needed to support preliminary observations identified in this review. Moreover, further post-mortem studies could be useful, especially if brain samples of various implicated anatomical regions could be obtained from a cohort of patients with accurately diagnosed neuropsychiatric disorders; one could then map out in greater detail the distribution of the relaxin-3 system in humans. The development of fully validated and characterized RXFP3 antibodies would markedly ameliorate the quality of such post-mortem studies. High-quality human studies along these lines would greatly benefit our understanding of this neuropeptidergic system in humans, possibly providing a robust foundation for eventual trials of therapeutic agents that target the relaxin-3/RXFP3 system.

**5. Conclusion**

In summary, this systematic review highlighted that the relaxin-3/RXFP3 system is likely involved, to some degree, in regulating behavioural correlates of depression and anxiety disorders. The comprehensive evaluation of animal-based pharmacological and genetic studies revealed a clear inconsistency among reported findings, due to several potential factors including heterogeneous study designs, site- and timescale- specific variability in interventions, and overlooked compensatory pathways. Nonetheless, some general trends were observed. The acute activation of RXFP3 generally had antidepressant and anxiolytic effects in several testing paradigms, and meta-analyses further demonstrated that such activation consistently increased 1- and 2-hour food intake in rats. Moreover, relaxin-3/RXFP3 KO studies in mice resulted in reduced circadian running wheel activity, indicative of diminished arousal behaviour. Our understanding of the relaxin-3/RXFP3 system could certainly benefit from additional animal studies probing the temporal and spatial deviations in behavioural modulation. However, it is of tantamount importance to conduct more human studies of relevance, particularly studies with strong methodological quality. It is hoped that further investigations along these lines will contribute to the development of future treatment options for neuropsychiatric conditions.

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**Declarations of Interest**

Gavin S. Dawe is employed by the National University of Singapore and is a co-inventor on patent applications on relaxin-3 B chain stapled peptide agonist and antagonists at RXFP3: “Stapled Peptide Agonists and Their Use in Treatment of Behavioural Disorders” Singapore Patent Application No. 10201709379P filed on 14 November 2017; and “Stapled relaxin-3 B chain peptide antagonists” Singapore Patent Application No. 10201904291Y filed on 13 May 2019.

Allan H. Young declares the following competing interests. Employed by King’s College London; Honorary Consultant SLaM (NHS UK). Paid lectures and advisory boards for the following companies with drugs used in affective and related disorders: AstraZeneca, Eli Lilly, Lundbeck, Sunovion, Servier, Livanova, Janssen, Allegan, Bionomics, Sumitomo Dainippon Pharma, COMPASS. Consultant to Johnson & Johnson. Consultant to Livanova. Received honoraria for attending advisory boards and presenting talks at meetings organised by LivaNova. Principal Investigator in the Restore-Life VNS registry study funded by LivaNova. Principal Investigator on ESKETINTRD3004: “An Open-label, Long-term, Safety and Efficacy Study of Intranasal Esketamine in Treatment-resistant Depression”. Principal Investigator on “The Effects of Psilocybin on Cognitive Function in Healthy Participants”. Principal Investigator on “The Safety and Efficacy of Psilocybin in Participants with Treatment-Resistant Depression (P-TRD)”. UK Chief Investigator for Novartis MDD study MIJ821A12201. Grant funding (past and present): NIMH (USA); CIHR (Canada); NARSAD (USA); Stanley Medical Research Institute (USA); MRC (UK); Wellcome Trust (UK); Royal College of Physicians (Edin); BMA (UK); UBC-VGH Foundation (Canada); WEDC (Canada); CCS Depression Research Fund (Canada); MSFHR (Canada); NIHR (UK). Janssen (UK). No shareholdings in pharmaceutical companies.

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