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Abstract: Fragile X Syndrome (FXS) is characterised by features including anxiety and autistic-like behaviour, which led to early hypotheses that aberrant physiological arousal may underlie the behavioural phenotype. In line with this, several lines of evidence suggest that the Hypothalamic-Pituitary-Adrenal (HPA) axis may be altered in the syndrome. This review collates evidence to determine the nature of HPA axis baseline activity and reactivity (as measured by glucocorticoid levels) differences in FXS, and its relationship to behaviour. Through a search electronic databases, 15 papers were identified which provided data on humans with FXS or the FMR1 knockout mouse model. The findings across studies are mixed, though trends in the findings can be seen, including elevations in cortisol levels, particularly in males. Preliminary findings also highlight associations between cortisol levels and key behaviours associated with the syndrome, such as gaze avoidance. Areas for future research are discussed.

Hypothalamic-Pituitary-Adrenal Axis Function in Fragile X Syndrome and its Relationship to Behaviour: A Systematic Review

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Abstract

Fragile X Syndrome (FXS) is characterised by features including anxiety and autistic-like behaviour, which led to early hypotheses that aberrant physiological arousal may underlie the behavioural phenotype. In line with this, several lines of evidence suggest that the Hypothalamic-Pituitary-Adrenal (HPA) axis may be altered in the syndrome. This review collates evidence to determine the nature of HPA axis baseline activity and reactivity (as measured by glucocorticoid levels) differences in FXS, and its relationship to behaviour. Through a search electronic databases, 15 papers were identified which provided data on humans with FXS or the FMR1 knockout mouse model. The findings across studies are mixed, though trends in the findings can be seen, including elevations in cortisol levels, particularly in males. Preliminary findings also highlight associations between cortisol levels and key behaviours associated with the syndrome, such as gaze avoidance. Areas for future research are discussed.

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Key Words

Fragile X Syndrome; FMR1; FMRP; autism; hypothalamic-pituitary-adrenal axis; cortisol; corticosterone; glucocorticoid; systematic review; KO mouse; HPA

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Response to Reviewers

Many thanks to the editor and to the reviewers for taking the time to kindly re-review the manuscript and provide useful feedback. I have addressed the issues raised and made amendments, as detailed individually below, using tracked changes:

As requested, I have re-structured the article to align to the review questions. I have, however, addressed gender differences according to each aspect of the cortisol release (baseline activity, magnitude/ duration of response, for humans and animals) following the discussion on that specific topic, and clearly marked this with sub-headings. However, I hope that I have now better highlighted the themes in gender differences observed, and have included a separate table on this issue, as requested.

When referring to each study in the text, I have briefly stated how many individuals were involved and what the ages of the individuals. I have done this for the studies with human participants, as from drafting this for the animal studies (which are addressed often is less detail in the text) this became very cumbersome to read. However, the animal results are presented directly alongside the sample sizes in Table 1, which I hope will make this interpretation more simple.

On p15 we mention the findings of Roberts et al (2009) in relation to the magnitude of cortisol responses. The authors of this study split their analyses according to two groups: those individuals with FXS who met the criteria for autism, and those who did not. The reviewer requested that this discussion be moved to the section on cortisol and behaviour. Given the paucity of studies discussing the magnitude of cortisol responses in this group, I have proposed that we leave this study here (as there is no option to present the results without referring to the grouping according to autism symptomatology) but to note that a further discussion on the topic is included later in the manuscript. Please do let me know if this is acceptable.

The first paragraph on page 17 was noted to be out of place. I have now clarified the intended point and moved this to the synthesis and future research section.

I have clarified through the text that Hessl et al (2002, 2006) and Hall et al (2006) studies utilised the same participants.

As requested, I have included a comment upon the difference in methodology (direct observation vs rating scales) in the Hall and Hessl et al studies, and it's potential relevance to the heterogeneous findings).

I have amended the terms "FXS-only" and "FXS+Aut" to "low levels of autism symptomatology" and "high levels of autism symptomatology".

I have clarified the sentence on p20 (relationships between cortisol and autistic symptomatology in syndromic and non-syndromic groups), as requested.

We hope that these revisions and the helpful feedback have satisfied your requirements, though please do get in touch with any further queries, and we would be happy to help. We look forward to publishing in your journal.

Kind regards,

Rebecca Hardiman & Alison Bratt

***Highlights**

- Preliminary findings, though mixed, suggest atypical HPA axis function in FXS
- Cortisol levels were associated with phenotypic behaviours, such as gaze avoidance
- Research is warranted to evaluate HPA axis function as a biomarker of autism in FXS

Hypothalamic-Pituitary-Adrenal Axis Function in Fragile X Syndrome and its Relationship to Behaviour: A Systematic Review

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Introduction

Fragile X Syndrome (FXS) is the most common known cause of inherited intellectual disability and the leading monogenetic cause of autism (Muhle, Trentacoste & Rapin, 2004; Turner, Webb, Wake, & Robinson, 1996), affecting approximately 1:4000 males and 1:8000 females (Sherman, 2002). Verkerk and colleagues (1991) categorized the genetic locus of the disorder as being an expanded CGG repeat on the long arm of the X chromosome, in the 5' untranslated region of the *FMR1* gene, occurring during maternal transmission. An expansion of 200 or more repeats typically causes the *FMR1* gene to become abnormally hypermethylated, silencing the production of the Fragile X Mental Retardation Protein (FMRP; Fu et al, 1991). FMRP is a ubiquitous transporter protein which carries target messenger ribonucleic acids (mRNAs; which contain genetic information) from the cell nuclei to ribosomes, where the information is decoded to produce specific amino acid chains for protein synthesis ((Feng et al., 1997; Irwin, Galvez & Greenough, 2000; Khandjian, Corbin, Woerly & Rouseau, 1996). The mRNAs served by FRMP have a broad range of purposes, though are largely involved in dendritic structure and function (Feng et al., 1997; Weiler et al., 1997).

The severity of the manifestation of the syndrome is variable, but individuals with FXS typically show marked behavioural features including deficits in attention, language and IQ; hyperactivity; anxiety; self-injury (particularly hand-biting); aggression; hyperarousal; stereotypies; and social difficulties, including gaze avoidance (Symons, Byiers, Raspa, Bishop & Bailey, 2010; Lachiewicz, et al., 1994; Bailey, Raspa, Olmsted & Holiday, 2008). The presentation of the FXS is quantitatively gender

dimorphic, due to the X-linked nature of the syndrome, with males typically (though not always) being more clearly affected than females. Crucially, anxiety plays a central role in many of the characteristic behaviours of the syndrome, including avoidance and confrontational behaviours (Sullivan, Hooper & Hatton, 2007) as well as autistic-like behaviour (Talisa, Boyle, Crafa & Kaufmann, 2014). It has long been hypothesised that aberrant or exaggerated physiological arousal, particularly stimulus-bound, may underlie these traits. Due to these tendencies for individuals with the condition to exhibit exaggerated behavioural responses to stressors, researchers have begun to investigate arousal and stress-related circuits and their relevance for individuals with FXS.

The hypothalamic-pituitary adrenal (HPA) axis is one of the body's main stress effector systems and is a circuit of interest in Fragile X research. Activation of the HPA axis triggers the release of glucocorticoids (such as cortisol in humans), which can be measured through blood or saliva sampling (Jessop & Turner-Cobb, 2008). Baseline release of cortisol follows a pronounced circadian rhythm marked by a peak after awakening, followed by a gradual decrease through the day, reaching a quiescent period during sleep (van Cauter, 1990): corresponding to the rest-activity cycle. Superimposed on this pattern, in response to physical or psychological stressors, is further pulsatile release of glucocorticoids, which is a normal, adaptive component of coping (Gunnar, 1987). In the short term the physiological changes associated with this ~~this induces~~ are adaptive in that they help to provide resources for successful coping, though enduringly high cortisol levels may have harmful effects (McEwen, 1998). Multiple negative feedback loops in the HPA axis exist to maintain adaptive levels of cortisol (Herman & Cullinan, 1997). In addition, when stressors are chronic, the HPA system may reach a

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stage of exhaustion (Selye, 1956) resulting in blunted cortisol levels and responses or even development of a pattern of decreases in response to stressors as a result of habituation (Miller, Chen & Zhou, 2007; Grissom & Bhatnagar, 2009).

Individual differences in HPA activity may also be important correlates or modifiers of social behaviour and behavioural and psychological responding to stressors. For instance, it has been noted that individuals with Cushing's syndrome (a condition characterised by chronically elevated baseline levels of cortisol) are more likely to experience negative psychological states, such as depression and anxiety (Kelly, Kelly & Faragher, 2003), suggesting that HPA hyper-activation may modify affect. In addition, research into shyness with participants of various ages suggests a complex functional interplay between cortisol and the regulation of social behaviour (for instance: Schmidt et al, 1997; Beaton et al, 2013; Kagan et al, 1988). Furthermore, there is a growing body of literature suggesting that individuals on the autism spectrum (which is characterised by atypical social behaviour) experience stress-related cortisol responses of increased magnitude and/ or duration (for instance: Spratt et al, 2012; Corbett, Mendoza, Abdullah, Wegellin & Levine, 2006), which may be driven by impaired negative feedback (Hoshino et al, 1987). Given the close association between FXS and autism spectrum disorder (ASD, for instance: Bailey et al, 1998), it will be important to consider autism symptomatology in the interpretation of FXS research.

In light of these associations between cortisol and behaviour, it is interesting that researchers investigating the FMRP target mRNAs have discovered an association between FXS and the HPA axis. *FMR1* knockout mice (an animal model of FXS) have been found to have fewer glucocorticoid receptors (GR-α) within neuronal dendrites, which would decrease homeostatic feedback regarding levels of cortisol (Jafari, Seese,

Babayan, Gall & Lauterborn, 2012; Miyashiro et al, 2003). Furthermore, in human subjects with FXS (but not typically developing or intellectually disabled controls), Annexin 1, a phospholipid-binding protein which mediates the inhibition by glucocorticoids on the HPA axis (Jessop, 1999), was synthesised and expressed abnormally (Sun, Cohen and Kaufmann, 2001). The level of dysregulation was closely associated with participants' level of FMRP, suggesting a direct regulatory relationship. Thus, it appears that lack of FMRP may result in excessive activation of the HPA axis, through impairing the negative feedback loop (Hessl et al, 2002). This highlights a pathway whereby cortisol regulation may be altered in FXS, which could play a direct or indirect relationship in the manifestation of the behavioural phenotype. Interestingly, broader studies of endocrine function in FXS, such as atypical negative feedback regulation of the thyroid (Bregman, Leckman & Rot, 1990) and cases of precocious puberty (Butler & Najjar, 1988; Moore, Chudley & Winter, 1990; Kowalczyk et al, 1996), support the presence of disturbances of the function of the hypothalamus and/or pituitary, which highlights another avenue to atypical cortisol regulation in the syndrome (Hessl, Riviera & Reiss, 2004).

There are also further features of FXS which may have relevance for the function of the HPA axis, a number of which we will review. Firstly, brain changes related to FXS may influence the emotional evaluation of events. Activation of the stress-effector systems relies on the evaluation of a stimulus or event by the limbic system: the "emotional centre" of the brain. One of the key components of this system, the amygdala, appears to be changed in FXS (Suvrathan & Chattarji, 2011). Broader excitatory and inhibitory imbalances in the FXS brain may also influence responding, in particular, a key glutamate receptor (MGluR5) which is affected in FXS plays an

important role in fear memory formation in the amygdala (Bear, 1998; Rodrigues et al, 2002). In turn, functional neuroimaging (fMRI) research has highlighted resultant atypical fear-specific functioning of the amygdala and a possible association between these brain changes and socioemotional deficits in individuals with FXS (Kim et al, 2012). These emotional-evaluative changes may clearly have downstream implications for cortisol release. Additionally, the gamma amino butyric acid neurotransmitter system appears to be commonly disordered in many neurodevelopmental disorders, including FXS (Braat & Kooy, 2015). Preclinically, FMRP has been shown to regulate GABA-ergic synaptic vesicle dynamics within the hippocampus of the Fmr1 KO mouse model, (Broek et al., 2016). Such genetically induced changes in the relative tonus of excitatory / inhibitory neurotransmitters within key limbic brain structures could potentially predispose to changes in stress responding. Finally, circadian rhythmicity (in terms of behaviour and biological clock component mRNAs in the liver) has also been shown to be deficient in mice lacking FMRP (Zhang et al, 2008); this broader disturbance of the biological clock may affect the pattern of activity in the baseline secretion of glucocorticoids via the HPA axis. The previous discussion highlights multiple ways in which HPA activity (as expressed in glucocorticoid levels) may be altered in FXS. In turn, activity in this system may directly modify and/or be indirectly associated with clinically significant behaviours in the syndrome (Hessl et al, 2002). As a consequence, research into the secretion of glucocorticoids has begun to emerge within the FXS literature. The aim of this review is to collate findings relating to HPA functioning in animal models of and humans with FXS. The inclusion of preclinical literature has been made in order to be able to conduct an in-depth analysis of the potential relationship between FXS and HPA function. The review addresses several questions:

a) Do individuals or animals with FXS exhibit atypical levels of glucocorticoids at baseline, or differences in the duration or magnitude of responses to stressors, compared to controls?

b) Given the X-linked nature of the condition, are there gender differences in the difference aspects HPA activity in FXS?

c) Do measures of HPA activity relate to behaviour, in individuals with FXS?

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Method

Selection Criteria for Studies

Types of studies. We considered relevant empirical or observational studies, written in English, which assessed measures of HPA output (cortisol in humans or corticosterone in mice, collected via salivary or haematological methods) in humans with full-mutation FXS or an animal model of the human full-mutation, such as the *FMR1* knock-out (KO) mouse. Papers were included if they contained either a group comparison of corticosterone levels or an analysis investigating the relationship between HPA activity and behaviour in individuals or animals with FXS. Case studies were considered when the individual's results were compared to normative data or matched with an individual without FXS.

Search Methods for Identification of Studies

Electronic search. The following databases were searched: Web of Science, SCOPUS, PubMed, and Academic Search Complete. The search was completed in June 2016.

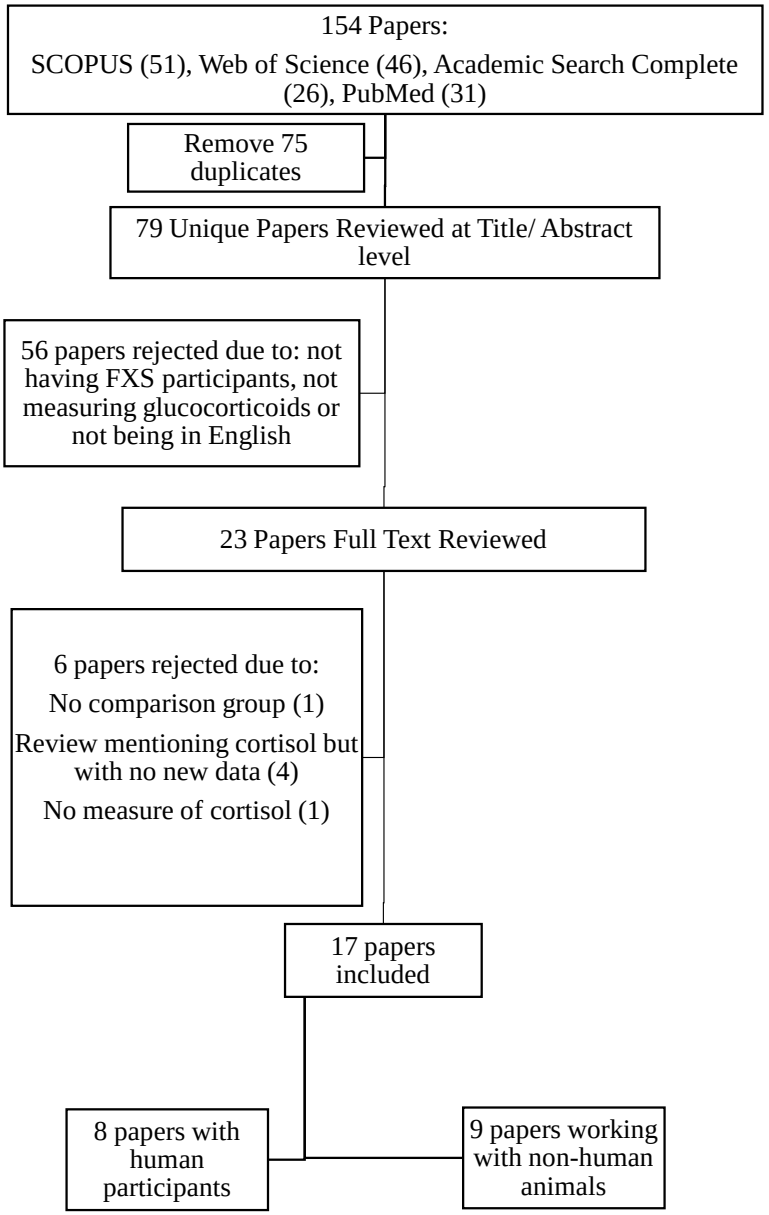
Search terms. The search terms used for the HPA axis search were: (("fragile x" OR *FMR1*) AND (glucocorticoid* OR cortisol OR corticosterone)). The fields 'title',

'abstract' and 'keywords' were searched (or closest available option within the database).

Searching other resources. Bibliographies of relevant articles were scrutinised. Furthermore, the titles of studies published in the following journals were searched, using the same terms, to ensure no papers had been missed in the database search: Psychoneuroendocrinology; American Journal of Medical Genetics; Journal of Intellectual Disability Research. These searches yielded no additional papers.

Search Results

The search is depicted in Figure 1. In total, 79 unique -papers were identified in the initial search, of which 17 met the inclusion criteria for this systematic review.



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~~a)~~ *Figure 1.* Depiction of the manuscript search process

Results and Discussion

Do Individuals or Animals with FXS Exhibit Atypical Levels of Glucocorticoids at Baseline, or Differences in the Duration or Magnitude of Responses to Stressors, Compared to Controls?

~~**Preclinical Literature: Results and Discussion**~~

Baseline HPA a~~A~~ctivity and circadian rhythm. ~~in Fragile X Syndrome Animal Models~~

Animal literature. Several studies (Table 1, includes summary of methodology and animal characteristics) have investigated the non-stressed corticosterone secretion of male KO mice compared to wild-type (WT) control animals, in order to identify whether changes exist in the baseline activity of the HPA axis in FXS animal models. The majority of studies found no genotype effect in their comparisons at single time-points, with male animals (Markham et al, 2006; Lauterborn, 2004; Nielsen et al, 2009; Eadie et al, 2009; Ghilan et al, 2015). Furthermore, in a more detailed analysis, Qin and Smith (2008) assessed the baseline circadian rhythm of both genotypes and found no difference at any of the six time-points tested (Qin & Smith, 2008). However, ~~T~~two studies did identif~~yied~~ genotype differences, though the nature of the difference contrasted: de Diego-Otero and colleagues (2009) found that KO mice had lower corticosterone levels at baseline than WT controls; in contrast, Qin and colleagues (2011) found a main effect whereby KO mice generally had higher corticosterone than WT controls. As such, there

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is no evidence to suggest baseline HPA activity is altered in males with FXS, based on the preclinical evidence.

Gender differences. No studies of this nature have utilised female animals, as such it is unclear whether any gender differences exist in this area (gender comparisons summarised in Table 4).

Human Literature. ~~**Diurnal Rhythm.**~~ Research investigating baseline HPA activity in humans has focussed upon profiling the diurnal rhythm of cortisol levels in this group (Table 3). Namely, ~~T~~two studies ~~(Wisbeck et al, 2000; Hessl et al, 2002)~~ investigated cortisol levels through routine days (without unusual or exciting events). Wisbeck and colleagues (2000) conducted a pilot study involving 7 females and 8 males (between the ages of 6-25 years) with FXS, comparing to a normative sample. Hessl and colleagues (2002) later built upon this with a larger study of 39 females and 70 males with FXS (age 6-17 years) compared to siblings without FXS (58 female, 51 male; age 6-17 years). In both studies, boys with FXS exhibited higher levels of cortisol, resulting from reduced diurnal decline, than their unaffected siblings. These findings may be consistent with the hypothesis, from preclinical literature on mRNA targets, of disordered HPA negative feedback. However, the only way to separate the direct influences of HPA feedback regulation and the influence of broader differences originating from, for instance, atypical emotional evaluation of the environment, would be to directly challenge the HPA axis, for instance with a dexamethasone suppression test (as used by Hoshino and colleagues (1987) with individuals with autism). As mentioned previously, heterogeneity in the preclinical literature in terms of both methodology and results means that it is challenging to draw conclusions about any potential results between the findings in mice and these suggestive findings of blunting

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of circadian glucocorticoid release in humans with FXS. Further investigations of circadian rhythmicity in the HPA axis in *FMR1* KO mice may help to establish further evidence to understand these observed differences better.

Gender differences. Of note, there are suggestive gender-differences apparent in the observations. Though no differences were observed in the initial smaller study (Wisbeck et al, 2000), in a later study, with larger numbers of participants (Hessl et al, 2002), visual analysis of the data revealed that the cortisol profiles of the females with FXS closely corresponded with those of their unaffected siblings: whereas the males showed more pronounced differences in cortisol levels. Of note, ~~though~~however, the statistical significance of these differences were not evaluated (gender comparisons summarised in Table 4). ~~A further discussion of gender-related differences is included below.~~

HPA r~~R~~eactivity to c~~C~~hallenges. Early hypotheses suggested that stimulus-bound arousal differences (Cohen, 1995) may play a significant role in the behavioural phenotype of FXS. Evidence to evaluate this claim has been collected across a small number of studies, involving both human and non-human animal participants. ~~in Fragile X Syndrome Animal Models~~

Animal Literature. Exposing animals to acute stress paradigms allows for investigation of the magnitude and/or duration of HPA axis reactions, and whether these differ in the *FMR1* KO model of FXS, compared to their WT counterparts. A commonly used trigger for acute stress with mice is to restrain the animal (for instance, in a small tube) for a period of time. A summary of this research is included in Table 1.

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Magnitude of response. Seven studies were identified which had compared the magnitude of responses of male KO and WT mice to this procedure (implemented for between 15 minutes and three hours; Table 1). Three of these studies found that KO mice exhibited higher levels of corticosterone compared to WT controls, following the stressor. De Diego-Otero and colleagues (2008) observed this difference following 15 minutes of restraint stress. In contrast, Lauterborn (2004) found a significant difference in corticosterone responses only after more prolonged restraint (2 hours), however only a trend towards a difference was observed with a shorter stressor (30 minutes). Ghilan and colleagues (2015) observed higher corticosterone levels after a short period of restraint (15 minutes) in the KO mice, compared to the WT mice. However, following more ~~significant increases in corticosterone after a short duration of restraint stress (15m) only in the KO mice, though after more~~ prolonged periods of restraint (30 and 60 minutes) both KO and WT mice showed responses which did not significantly differ in magnitude. Increased stress-related elevations were also seen in response to a different stressor (spatial novelty) by Romero-Zerbo and colleagues (2009), who found that, despite initially lower baseline corticosterone levels in the KO mice, following stressors the KO mice exhibited higher levels of corticosterone than their WT counterparts.

In contrast to the four studies finding elevations in KO mice responses, Eadie and colleagues (2009) found that KO mice had significantly lower corticosterone than WT, following 3 hours of restraint stress, suggesting a smaller hormonal response to the paradigm. Furthermore, there were no genotype differences observed in seven studies: three studies did not observe any genotype difference in the magnitude of corticosterone responses to restraint stress (Markham et al, 2006; Nielsen et al, 2009; Qin & Smith, 2008) and a further four studies also observed no difference using other

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acute stress paradigms, including exposure to spatial novelty (Nielsen et al, 2009; Qin & Smith, 2008; Qin et al, 2011) and swim stress (Nielsen et al, 2009). ~~Furthermore, the only study to use female animals (Markham et al, 2006) found no difference in peak responding following physical restraint for 30 minutes.~~

Of interest, given the atypical social profile associated with Fragile X, de Diego-Otero and colleagues (2008) investigated the mice's reactions to both physical (restraint) and social stressors (housing with between 9 and 11 other animals for 15 minutes), to investigate whether there may be differences in the nature of corticosterone responses. They found that ~~, in contrast to the increased response of KO in response to restraint stress,~~ KO mice showed lower levels of corticosterone following the social stressor than WT mice, which differs from the trend for elevations in response to restraint stress in other studies. This preliminarily suggests that the nature of the stressor (social verses physical) may be of importance when investigating stress-related physiology in the FXS mouse model. Finally, ~~A~~another interesting manipulation was included in a study by Qin and colleagues (2011) who exposed both WT and KO animals to chronic restraint stress, before exposure to an acute stressor in the form of a novel environment (Qin et al, 2011). However, no interaction was found between the genotype and chronic stress, on the corticosterone responses.

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In summary, given the high numbers of null findings no firm conclusions can be drawn about the magnitude of responses in FXS mouse models. Where differences were observed, however, the trend was for male animals to exhibit higher levels of corticosterone. A possible reason for this variation in results between studies may be related to the genetic background of the mice used. Mouse strain differences have been previously found to influence both the magnitude and duration of corticosterone

responses to stressors (Shanks, Griffiths, Zalcman, Zacharko & Anisman, 1990) and have been hypothesised to be associated with conflicting results more broadly, when using the *FMR1* KO (Pietropaulo, Guilleminot, Martin, Amato & Crusio, 2011). Interestingly, Markham and colleagues (2006) found male KO mice had protracted responses to 30 minutes of restraint when compared to WT mice, using mice of a C57/Bl6 background; however Qin and Smith (2008) did not find any genotype differences after the same stressor when using FVB/NJ male mice. However, clearly, there may have been other methodological differences between the studies which caused the differences in the results (see Table 1 for summary of key study methodology). For instance, the timings of the testing of the animals (when specified) varied between 7am and 2pm. The active phase of mice is typically during the night time, inverse to humans, with a peak at approximately 8pm (Gong et al, 2015), though of course housing and lighting conditions may cause this to vary. The time widows for testing across the reviewed studies overlapped substantially making comparisons challenging. However, this possible influence should be considered in future research and there is a need to establish better evidence on the link between sample timings, circadian rhythmicity and stress-related corticosterone release in *FMR1* KO mice, in order to facilitate the interpretation of the literature.

Duration of response. Next, several studies have investigated the duration of corticosterone responses. This was achieved by conducting timecourse studies involving sacrificing groups of mice at differing lengths of time following a restraint stressor. Interestingly, Markham and colleagues (2006) observed that the male KO animals showed a slower return to unstressed baseline than WT; a pattern which is consistent with the prediction of reduced HPA negative feedback. Though, two other

studies did not find any genotype differences between male animals in response duration (Nielsen et al, 2009; Qin & Smith, 2008).

Gender differences. One study was identified which included female mice (Markham et al, 2006; gender comparisons summarised in Table 4). In this research, no difference was found in peak responding between male and female KO mice, following physical restraint for 30 minutes. However, female KO mice ~~Female animals in Markham and colleagues' study (2006)~~ showed a different pattern of response and recovery to their male counterparts, but also atypical compared to the WT mice: the female KO mice appeared to show a protracted rise, as their the peak corticosterone level was at the final 60m sample, where levels would be expected to be falling (there were no gender differences in the WT animals). This suggests that animal gender may play an important role in the outcomes of research into HPA output in FXS. However, further research is needed.

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~~Next, several studies have investigated the duration of corticosterone responses. This was achieved by conducting timecourse studies involving sacrificing groups of mice at differing lengths of time following a restraint stressor. Interestingly, Markham and colleagues (2006) observed that the male KO animals showed a slower return to unstressed baseline than WT; a pattern which is consistent with the prediction of reduced HPA negative feedback. Though, two other studies did not find any genotype differences between male animals in response duration (Nielsen et al, 2009; Qin & Smith, 2008). Female animals in Markham and colleagues' study (2006) showed a different pattern of response and recovery to their male counterparts, but also atypical: the female KO mice appeared to show a protracted rise, as their the peak corticosterone~~

~~level was at the final 60m sample, where levels would be expected to be falling (there were no gender differences in the WT animals).~~

~~Finally, there has been no research to date investigating whether individual differences in corticosterone responses relate to differences in behaviour. Though, it is unclear how such research may translate to understanding of human behaviour as the behavioural phenotype of the mouse model does not correspond closely to that of the human phenotype. Namely, in comparison to the increased levels of anxiety associated with FXS in humans (for instance; Cordiero et al, 2011), all studies which utilised a behavioural assay to assess anxiety (Elevated Plus Maze; Pellow, Chopin, File & Briley, 1985) observed that *FMR1* KO mice exhibit decreased behavioural indicators of anxiety relative to their WT counterparts (Qin et al, 2011; de Diego-Otero et al, 2008; Qin & Smith, 2008; Eadie et al, 2009). No research has been conducted to identify which mouse behaviours correspond to clinically significant behaviours in FXS. However, should these be identified, animal models may help to highlight relationships between HPA axis function and behaviour in FXS.~~

~~Human Literature: Results and Discussion~~

~~Between-Group Comparisons of Cortisol Secretion in Humans~~

~~***Human literature.*** Four studies conducted between-group analyses to compare the release of cortisol by individuals with FXS to other groups (see Table 2 for details of study participant characteristics and Table 3 for details of between-group comparisons).~~

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~~**Diurnal Rhythm.** Two studies (Wisbeck et al, 2000; Hessl et al, 2002) investigated cortisol levels through routine days (without unusual or exciting events). In both~~

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studies, boys with FXS exhibited higher levels of cortisol, resulting from reduced diurnal decline, than their unaffected siblings. These findings may be consistent with the hypothesis, from preclinical literature on mRNA targets, of disordered HPA negative feedback. However, the only way to separate the direct influences of HPA feedback regulation and the influence of broader differences originating from, for instance, atypical emotional evaluation of the environment, would be to directly challenge the HPA axis, for instance with a dexamethasone suppression test (as used by Hoshino and colleagues (1987) with individuals with autism). As mentioned previously, heterogeneity in the preclinical literature in terms of both methodology and results means that it is challenging to draw conclusions about any potential results between the findings in mice and these suggestive findings of blunting of circadian glucocorticoid release in humans with FXS. Further investigations of circadian rhythmicity in the HPA axis in *FMR1* KO mice may help to establish further evidence to understand these observed differences better.

Of note, there are suggestive gender differences apparent in the observations. Though no differences were observed in the initial smaller study (Wisbeck et al, 2000), in a later study, with larger numbers of participants (Hessl et al, 2002), visual analysis of the data revealed that the cortisol profiles of the females with FXS closely corresponded with those of their unaffected siblings; whereas the males showed more pronounced differences in cortisol levels. Of note, though, the statistical significance of these differences were not evaluated. A further discussion of gender related differences is included below.

Cortisol Reactivity. Early hypotheses suggested that stimulus-bound arousal differences (Cohen, 1995) may be significant in FXS. Evidence to evaluate this claim has

~~been collected in F~~four studies to date~~, which~~ have investigated group differences in the release of cortisol in response to cognitive, behavioural or physical testing (see Table 2 for details of study participant characteristics and Table 3 for details of between-group comparisons). Preliminary evidence for atypical regulation is provided by a case study of an adult male (age 24 years) with FXS who showed an atypical pattern of adaptation in response to physical exercise: an early increase in cortisol followed by a large decrease, opposite to the pattern of adaptation seen in the healthy controls (15 males; Bricout et al, 2008).

Larger studies have also evaluated differences in the magnitude of cortisol reactions, focussing particularly on the response to social stressors (due to the atypical social behaviour associated with the syndrome). The findings of these studies are mixed. Firstly, Hessl and colleagues (2002) observed that males with FXS (70, age 6-17 years) showed reduced diurnal decline in the period after meeting unfamiliar researchers, compared to the siblings (58 female, 51 male, age 6-17 years), which the authors suggested may have resulted from an increased response to this social challenge. In addition, Scherr and colleagues (2016) found that, in the first year of the longitudinal study, boys with FXS (31, age 9-14 years) showed ~~reduced decline between a 9am and 12pm sample (during which time an assessment battery was administered),~~ higher levels of reactant cortisol following an assessment battery, when compared to TD controls (49, matched on non-verbal mental age, 4-9 years). These differences were not observed in the following two assessment years, in which fewer individuals participated (Scherr, 2013). ~~Visual analysis suggested that the baseline levels of cortisol increased over the years of assessment in the FXS group, but not the TD group. Though the~~In addition, levels of baseline cortisol were higher in the FXS group than the comparison

group, though this ~~e group~~ difference did not reach a level of statistical significance. In addition, the authors noted differences in the changes of cortisol levels over the longitudinal assessment. Firstly, ~~The study authors also noted that~~ the degree of change in cortisol levels over the years of the longitudinal assessment (reactant minus baseline levels) increased in the FXS group, as compared to the TD controls. Visual analysis suggested that the baseline levels of cortisol increased over the years of assessment in the FXS group, but not the TD group. As such, the evidence from these two studies, as well as the aforementioned case study, suggest possible differences in the responses of boys with FXS, as well as differences in the development of this regulation over time.

However group differences were not observed in all studies. Further analysis~~A later study~~ of the data collected in the study by Hessl and colleagues (2002), ~~by Hessl and colleagues (2006) with the same sample as the aforementioned study by the same team (Hessl et al, 2002)~~did not find any differences between the children with FXS and unaffected siblings in cortisol levels in response to, or following, a structured social challenge (Hessl et al, 2006. FXS group: 58 males, 32 females, age 6-17 years. Sibling group: 53 females, 37 males, age 6-17 years). Finally, Roberts and colleagues (2009) conducted an evaluation of 51 males with FXS (mean age 3 years) compared to 21 male TD controls (mean age 4 years) investigated the magnitude of cortisol responses to a social interaction between children with FXS and their siblings without FXS, though divided the FXS group according to degree of autism symptomatology in their analysis. It was found that, although there were no differences between young boys with FXS and low levels of autism symptomatology ~~only~~ (who did not meet the criteria for a dual diagnosis of autism spectrum disorder on the CARS) and their siblings, children with FXS ~~+ASD~~and high levels of autism symptomatology had higher levels of cortisol both

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prior to and following social interactions with an unfamiliar experimenter (though there were no differences in the magnitude of the response). This suggests that there may be differences in cortisol profiles within the population of people with FXS, relating to the degree of autistic symptomatology. The relationship between cortisol and autism symptomatology is discussed in further detail later in this review.

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Thus, as with the findings in the preclinical literature, the findings of the studies in humans are heterogeneous. However, where differences were observed between the 'typical' or baseline cortisol levels of individuals with and without FXS, they were manifested as relative increases, rather than decreases, in cortisol secretion. This corresponds to the preclinical observations of comparatively higher corticosterone responses to stressors in *FMR1* KO mice in four studies; though, as mentioned above, seven studies found no genotype difference in these animals. However, this potential trend in the findings highlights an avenue for future investigation.

Gender differences. Given the broad gender differences in the manifestation of FXS, researchers have chosen to investigate whether there are differences in cortisol responses between males and females with FXS, in four studies. In two studies, it was observed that males showed higher levels of cortisol following social challenges (a brief social stressor: Wisbeck et al, 2000; interaction with an unfamiliar experimenter: Hessl et al, 2002) than females. This suggests that atypical responding may be limited to, or at least exaggerated, in males with FXS, compared to females with the condition, mirroring the observations in the preclinical literature on the topic. Of note, however, the differences in both studies were based on visual observances and were not statistically evaluated. In contrast, both Hessl and colleagues (2006) and Hall and colleagues (2008:

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29 females and 31 males, age 5-20 years) found no gender differences in their studies, where statistical comparisons were conducted. Namely, Hessl and colleagues (2006) found no differences in the magnitude of response to a social challenge and Hall and colleagues (2008) saw no differences in diurnal decline across a day which involved unfamiliar social interactions in the form of evaluations by the experimenters.

Therefore, the results between studies are mixed, which may, in part, reflect the higher variability in the presentation of FXS in females, resulting from processes such as X-inactivation. Though, in the wider literature, there is evidence of gender-related differences in HPA in adulthood, though it is unclear whether robust differences exist in younger individuals (Jessop & Turner-Cobb, 2008), such as those included in the studies in FXS. It is possible that there are also FXS-independent differences which contribute to this gender dimorphism. More detailed exploration of the relationship between other biomarkers (such as FMRP), cortisol and behaviour in males and females with FXS may help to clarify the origins of this variability and verify whether differences do exist.

~~Given that individuals with FXS are prone to experiencing anxiety or phobias relating to many, varied situations (Cordeiro, Ballinger, Hagerman & Hessl, 2011), it is possible that idiosyncratic circumstances (outside of the examined social challenges or interactions) may trigger atypical cortisol responses. Of note, individuals with autism have been shown to have differential patterns of reactions to social-evaluative and non-social (such as unpleasant sensations) stimuli (Taylor & Corbett, 2014). Individuals with FXS are known to experience atypical sensory processing (Belser & Sudhalter, 1995) and have been shown to show elevated startle responses to sensory stimuli (Miller et al, 1999). Therefore, future research should address cortisol responses to a wider variety of situations which may be challenging for individuals with FXS.~~

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Is there a Relationship Between Cortisol Levels and Behaviour ~~in~~ within Fragile X Syndrome?

Animal literature. ~~Finally,~~ To date, there has been no research to date investigating whether individual differences in corticosterone responses relate to differences in behaviour. Though, it is unclear how such research may translate to understanding of human behaviour as the behavioural phenotype of the mouse model does not correspond closely to that of the human phenotype. Namely, in comparison to the increased levels of anxiety associated with FXS in humans (for instance; Cordiero et al, 2011), all studies which utilised a behavioural assay to assess anxiety (Elevated Plus Maze; Pellow, Chopin, File & Briley, 1985) observed that *FMR1* KO mice exhibit decreased behavioural indicators of anxiety relative to their WT counterparts (Qin et al, 2011; de Diego-Otero et al, 2008; Qin & Smith, 2008; Eadie et al, 2009). No research has been conducted to identify which mouse behaviours correspond to clinically significant behaviours in FXS. However, should these be identified, animal models may help to highlight relationships between HPA axis function and behaviour in FXS.

Human literature. Five studies conducted within-group comparisons to investigate the relationship between salivary cortisol and measures of behaviour in individuals with FXS (see Table 2 for participant details and Table ~~5~~4 for study details).

Social and Autistic Behaviours. Many people with FXS display autistic-like characteristics including: gaze-avoidance, repetitive behaviour and shyness. However, not all individuals with FXS display levels of autistic symptomatology which mean that

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they meet the diagnostic criteria for an autism spectrum disorder (Talisa et al, 2014). As such, a number of studies have investigated possible factors associated with the degree of autism symptomatology, including salivary cortisol.

Three studies ~~Three studies collected~~which have utilised observational measures of behaviours exhibited by individuals with FXS, during various types of social interaction. ~~, and examined them in relation to salivary cortisol.~~ Two of these studies, utilising the same group of participants, ~~,~~observed the behaviour of individuals with FXS during a structured social challenge~~s~~, which involved asking the child to read, answer questions and sing in front of others (Hall et al, 2006~~;~~ Hessl et al, 2006). Many of the measured behaviours were not found to have relationships with cortisol levels including: vocal quality (including mumbling or intrusive tones: Hessl et al, 2006) discomfort (participant appears in crisis, demonstrating behaviours such as self-injury, crying, aggression: Hessl et al, 2006. Hand-biting was also assessed separately in: Hall et al, 2006) non-verbal task avoidance (physically leaving the situation or covering eyes; Hessl et al, 2006; Hall et al, 2006), verbal refusals (Hall et al, 2006). Though, a positive correlation was observed with fidgeting (Hall et al 2006). Most interestingly, however, gaze avoidance, one of the characteristic features of the FXS phenotype, was found to relate to levels of cortisol in both studies, though the direction of the associations differed. Hessl and colleagues (2006) found that (across males and females with FXS), after controlling for other potential influences on cortisol levels, increased gaze aversion was associated with a lower post-challenge levels, to the social challenge. In fact, it was noted that the most gaze aversive children exhibited decreases in eye contact in response to the challenge. In contrast, Hall and colleagues (2006) found that *increased* mean levels of cortisol were associated with decreased eye contact.

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~~T~~However, these findings raise two hypotheses as to whether the primary influence on gaze avoidance relates to autistic-like characteristics (i.e. a lack of response to social stimuli) or social anxiety (i.e. an excessive response to social stimuli; Hessl et al; 2006). Both hypotheses are interesting and warrant further investigation.

Furthermore, the relationship between salivary cortisol and social approach behaviour during naturalistic social interactions has been explored in one study (Roberts et al, 2009). The method involved investigating social approach behaviour (physical approach, facial expressions and eye contact) with an experimenter when they were both unfamiliar (first minute of interaction) and familiar (during last hour of day-long assessment) to the child. Typically, as also seen in the controls in this study, children who approach an unfamiliar person more show an increased reaction and those who later approach the experimenter more (when familiar) initially had higher baseline levels of cortisol. However, the children with FXS showed a different pattern of association in this study. Firstly, the participants with FXS and low -levels of autism symptomatology ~~(FXS only)~~ showed no significant association between cortisol and behaviour at all. Whereas, within the group of children with FXS and high levels of autism symptomatology~~+ASD~~, boys with higher cortisol levels (following the interaction) showed fewer physical approaches to the unfamiliar experimenter: the opposite pattern to in the control group. As such, this study suggests a possible association between heightened physiological reactions to social situations, and increased social avoidance.

Further evidence on the association between cortisol and autistic behaviour in FXS comes from studies which have utilised broader autism screening or diagnostic measures. ~~Autistic-like characteristics form a key part of the FXS behavioural phenotype~~

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~~and, as such, have been of focus in the research on HPA associations.~~ Hall and colleagues (2008) utilised a direct observational assessment measure (ADOS-G; Lord, Rutter, DiLavore, & Risi, 2002) with their participants. The results of the study indicated that lower baseline levels of cortisol were associated with higher levels of autistic behaviour. Hessl and colleagues (2006) also found a relationship between cortisol and some types of autistic behaviour: increased sensory and social relation problems were positively associated with cortisol reactivity to a social challenge. Roberts and colleagues (2009), in contrast, found that *reduced* cortisol reactivity (which the authors suggest could be related to elevated basal levels) to a social interaction was associated with increased autistic behaviour (as measured on a behaviour rating scale: CARS; Schopler, Reicher & Renner, 1980), only within the group of individuals with FXS~~+ASD~~ and high levels of autism symptomatology; in the group of children with FXS and low levels of autism symptomatology~~ only~~, there was no relationship between cortisol and levels of autistic behaviour.

As such, a number of studies highlight associations between HPA activity and this key part of the FXS behavioural phenotype. However, the nature and direction of this association varies, with some finding increased levels of cortisol to be associated with increased autism symptomatology, both other with decreased cortisol levels. The heterogeneity of measures behaviour (direct observation as compared to informant rating scales) and cortisol may underlie such differences. In addition,

~~T~~the findings of Roberts and colleagues (2009) raise the possibility that levels of cortisol more strongly relate to behaviour in individuals with FXS and high autism symptomatology, as compared to those with lower symptomatology. In fact, the authors suggest that HPA dysregulation may serve as a biomarker of ASD in FXS. This highlights

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that individuals' levels of autistic behaviour may be important to consider when interpreting the results of studies of the relationship between cortisol and behaviour in FXS. Though, variations in the assessment of autistic behaviour across the other reviewed studies make it challenging to evaluate this further based on the existing evidence. Future research to study the gradation of ASD in FXS would be valuable to ~~delinate~~delineate phenotypic boundaries and evaluate the significance of HPA function as a biomarker of ASD in FXS.

There is a growing body of literature relating to idiopathic autism which is also of relevance to this discussion. A review of this literature, revealed differences in both HPA rhythm and responsiveness in individuals with autism (Taylor & Corbett, 2014). Typically, in response to social situations, individuals with autism exhibit blunted responsiveness, which corresponds to the patterns seen by both Hall and colleagues (2008) and Hessl and colleagues (200~~6~~9). Interestingly, however, the differences observed in those with idiopathic autism seem to be moderated by levels of functioning: there is not conclusive evidence that HPA dysregulation observed in lower functioning individuals also applies to individuals with high functioning autism~~)~~. In Roberts and colleagues' study, the participants with both FXS and ~~ASD~~ high levels of autism symptomatology had lower levels of adaptive behaviour than those with ~~FXS only~~low levels of autism symptomatology (though the significance of the difference was not evaluated), highlighting a potential confound. Future research should examine this potential relationship in the FXS population.

A broader question relating to the question of the association between autistic behaviour in FXS is the debate as to whether autistic-like behaviours in individuals with FXS meaningfully correspond to the characteristics seen with idiopathic autism (Hall et

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al, 2010). It is possible that autistic-like behaviours in FXS have different causal mechanisms and, as such, the relationships between cortisol and behaviour may differ in those with syndromic and non-syndromic autistic characteristics. Future research might help to address these issues. For instance, ~~C~~comparison of the relationships between cortisol and behaviour in those with autism, including those with non-syndromic autism and those with FXS who meet the criteria for autism, ~~with and without FXS~~ may help to elucidate whether cortisol and behaviour relations differ in their nature or development, dependent upon genetic status.

Behaviour ~~p~~Problems. Behavioural problems and challenging behaviours are a key issue of concern for many caregivers of people with FXS. Such behavioural issues are often anecdotally reported to be related to 'hyperarousal', meaning that objective investigations between these behaviours and physiological responses are warranted. In order to explore this issue, ~~T~~two studies have utilised the Child Behaviour Checklist (CBCL; Achenbach, 1991) as a broad measure of behaviour problems, and explored relations between scores and ~~with~~ cortisol levels. Hessl and colleagues (2002; ~~.)~~ ~~investigated the associations between cortisol and CBCL scores (~~controlling for other factors which were found to be predictive of the scores) ~~and~~ found that a composite score of cortisol significantly predicted 14% of the variance in total behaviour problem scores for females with FXS. Further analyses indicated that increased cortisol levels were specifically associated with increased social and attention problems. Furthermore, a composite score representing cortisol secretion on typical days significantly positively correlated with attention problems and approached significance for somatic complaints and social problems. There were no other relationships between cortisol and other of the measured behaviour problems, including: withdrawn behaviour, anxious or

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depressed behaviour, thought problems, aggressive behaviour or delinquent behaviour. In the same study cortisol levels accounted for 8% of variance in total behaviour problems in the males with FXS, which approached significance. The strongest association with a subscale score was with withdrawn behaviour. In comparison, in a later study ~~by the same team~~with the same participants (Hessl et al, 2006), no ~~found no~~ relationship was found between CBCL scores and any cortisol measures (baseline, post-challenge cortisol or magnitude of change) taken in relation to a social challenge.

Other Characteristics. Scherr and colleagues (2016) found that increased baseline levels of cortisol were associated with lower verbal working memory performance in boys with FXS, suggesting a possible link between arousal levels and academic-related performance.

Synthesis and Future Research

There are some interesting preliminary findings in this existing research. Though the findings are heterogeneous, there are some interesting observations and trends within the relatively few studies that have addressed the issue of HPA function in FXS, to date. In mice, no robust differences in baseline cortisol levels were seen, though there was some evidence of elevated stress-related reactivity. In human studies, baseline differences were observed in several studies (Hessl et al 2002; 2006; Roberts et al, 2009), as well as some indications of reactivity differences, compared to TD children (Scherr et al, 2016; Hessl et al, 2002; Roberts et al, 2009), though such differences may be mediated by gender and degree of autism symptomatology. At present, specific conclusions about the role of cortisol levels in behaviour associated with FXS are difficult to draw due to the high levels of variability and lack of correspondence between studies. However, there are suggestions of associations between cortisol levels

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and autistic behaviour, behaviour problems and key cognitive processes (working memory). Future research will undoubtedly help to clarify some of these uncertainties and strengthen the evidence to clarify the robustness of the observed themes.

In addition to the suggestions discussed through the previous sections, there are several other considerations for future projects. The wider information on the HPA system highlights its complexity, with individual differences relating to multiple factors, including: medication, pubertal stage, gender, temperament, chronic stress, compliance with the sampling protocol, nature of stressors, familial genetics and BMI (Jessop & Turner-Cobb, 2008; Gunnar et al, 2003; Dickerson & Kemeny, 2004; Kupper et al, 2005). As well as the variation in study methodology, many of these potential influences have not been explored or accounted for in the research and may relate to the observed variability in study findings. Furthermore, the research to date has provided important but limited snapshots of the activity of the HPA axis, with the exception of Scherr and colleagues' (2016) longitudinal study, in small groups of individuals. Prospective or longitudinal studies including further information about an individual's characteristics, behaviour, environment and biology would help to provide a more detailed picture of the role of HPA activity in this population.

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In addition, research involving humans has utilised TD comparison groups. However, an important step in future research will be to investigate the specificity of any group differences to individuals with FXS. It is possible that, rather than being directly FXS-related, the differences observed could relate to having an intellectual disability or autistic behaviour, and may be seen in others without FXS, but with similar characteristics. For instance, earlier research with people with autism has highlighted that level of cognitive functioning relates to the findings: with differences being

observed predominantly in those described as "low functioning" (Hoshino et al, 1987; Corbett et al, 2006; Richdate & Prior, 1992; Taylor & Corbett, 2014). In addition, given the aforementioned potential link also between levels of autism symptomatology ~~autism~~ and cortisol levels in those without FXS, and the high levels of autistic behaviour seen in people with FXS (Clifford et al, 2007), this clearly may be a confound in the differences seen. This is particularly pertinent given the findings of Roberts and colleagues (2009), who found that cortisol differences in FXS, compared to TD controls, was dependent upon levels of autism symptomatology ~~autism status~~. Thus, future research should employ control groups to help address these potential confounds, including those with non-syndromic autism and those with idiopathic intellectual disabilities.

In addition, in the human literature discussed above, much of the focus has been upon investigating HPA responses relating to social interactions or demands, in individuals with FXS. Given that individuals with FXS are prone to experiencing exaggerated behavioural responses, anxiety or phobias relating to many, varied situations (Cordeiro, Ballinger, Hagerman & Hessl, 2011), it is possible that idiosyncratic circumstances (outside of the examined social challenges or interactions) may also trigger ~~atypical~~ cortisol responses that differ in magnitude or duration, compared to the general population. For instance, individuals with FXS are known to experience atypical sensory processing (Belser & Sudhalter, 1995) and have been shown to show elevated startle responses to sensory stimuli (Miller et al, 1999). Of note, research with individuals with autism has highlighted ~~have been shown to have~~ differential patterns of reactions to social-evaluative and non-social (such as unpleasant sensations) stimuli (Taylor & Corbett, 2014). ~~Individuals with FXS are known to~~

~~experience atypical sensory processing (Belser & Sudhalter, 1995) and have been shown to show elevated startle responses to sensory stimuli (Miller et al, 1999).~~ Therefore, future research should address cortisol responses to a wider variety of situations which may be challenging for individuals with FXS, in order to gain a broader picture of HPA activity in this population, and it's potential applicability to day-to-day challenges.

Finally, given the possible significance of arousal differences, as evidenced by potential links with cognition and behaviour, researchers should also evaluate potential strategies for managing levels of arousal and systematically assess for any resultant improvements more widely. Scherr and colleagues (2016) highlight that individuals with FXS may benefit from targeted arousal-reducing interventions, such as the teaching of coping skills or relaxation techniques. This could include, for instance, the use of mindfulness-based techniques, which have been shown to reduce levels of arousal in individuals with Williams Syndrome (~~Miodrag et al., 2013~~Miodrag et al., 2013). There has been a paucity of systematic evaluations of potential interventions for arousal issues in individuals with FXS, which include physiological measures, such as assessment of cortisol. However, Hall and colleagues (Hall, Lightbody, McCarthy, Parker, & Reiss, 2012) piloted intranasal oxytocin as an intervention for social anxiety in a small group of boys with FXS and found both increases in eye contact and decreases in cortisol levels after administration. This suggests that there may be avenues for both pharmacotherapy and behavioural interventions when seeking ways to support individuals with FXS in this area.

Conclusion

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In summary, there is emerging evidence that cortisol levels differ in individuals with FXS compared to TD controls and relate to socially significant behaviours, thus highlighting a number of important avenues for future exploration. Delineating the significance and role of HPA activity in the syndrome will help to further our understanding of the mechanisms of the condition and may lead to provision of more effective support.

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Table 1

Studies investigating corticosterone secretion in FMR1 knockout mice.

Study	Cort. measure	Method Sacrificed	Gender (M/F)	Mice per group	Strain	Age of Mice	Basal measure	Stress Condition(s)	Recovery Time	Time Tested	Cort. Findings
Ghilan et al (2015)	Blood serum from trunk	Decapitation following anaesthetisation by isoflurane	M	7-18	C57Bl/6	55-65d	/	Restraint (conditions: 15m/ 30m/ 1h) or control	None: quick sacrifice after restraint	9am-11am	WT mice showed significant elevations only after 30m or 1h of restraint. KO mice showed increases after all restraint periods. After 15m restraint, KO mice significantly higher corticosterone than WT. Suggests even short stress exposures trigger response in KO mice

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de Diego-Otero et al (2008)	Blood plasma	Retro- orbital puncture	M	8-12	FVB-129	60-180d	/	Social stress (15m) or acute immobilisation stress (15m) or control	None: immediate sacrifice following behavioural . test battery	-	KO in control and social stress conditions lower corticosterone than WT. Acute stress KO higher corticosterone than WT
Lauterborn (2004)	Blood plasma from right ventricle	Overdose with euthansol	M	-	FVB*	-	/	Restraint (30m/ 2h) control	-	10am-2pm	Following 2h restraint KO higher corticosterone than WT, similar ns trend following 30m restraint
Markham et al (2006)	Blood serum from trunk	Rapid decapitation	M & F	8-12	C57/Bl6	40-45d	Cagemate sham comparisons (no restraint, just moved to test room)	Restraint (30m) or control	Conditions: 0/ 15/ 60m	10am - 12.30 pm	Male KO protracted return to unstressed baseline (still elevated at 60 m). Female show protracted rise compared to WT. peak secretion does not differ between

											genotypes.
Nielsen et al (2009)	Blood plasma from trunk	Rapid decapitation	M	5-12	FVB/NJ x C57/Bl6 (F1 hybrid)	11-12w	/	Swim Stress (3m) or open field (10m) or restraint (unspecified length). Each condition with control.	Swim: 17m. Open field: 10m. Restraint conditions: 0/ 30/ 60/ 90/ 120m	7am-9am	No genotype difference in magnitude or duration of corticosterone response to any stressor.
Qin, Xia, Huang & Smith (2011)	Blood plasma from trunk	Rapid decapitation	M	19-24	FVB/NJ	96±1d	/	Prior stress: chronic stress (2h/d restraint x10) or control. Acute stressor: spatial novelty (EPM)	-	-	No interaction between genotype and chronic stress condition. Main effect genotype: corticosterone higher in KO.
Qin & Smith (2008)	Blood plasma from trunk	Rapid decapitation	M	10-12	FVB/NJ	100±10 d	2am, 6am, 10am, 2pm, 6pm, 10pm	Acute restraint stress (30/ 120 m) or spatial novelty (EPM 5m) or control	Conditions: 30/ 120m	Before 11am	WT and KO no circadian rhythm differences (basal measures). Following stressors, no genotype difference in any

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										condition
Eadie et al (2009)	Blood plasma from trunk.	Rapid decapitation	M	4	C57BL/6	-	Acute restraint stress (3 hours) or control	Immediate sacrifice following stressor	9am-1pm	No difference in control condition but following stressor NO showed significantly lower corticosterone.
Romero-Zerbo et al (2009)	Blood serum	Cervical dislocation	M	10-11	FVB-129	90-120d	Open field	Immediate sacrifice following stressor	-	At baseline, KO significantly lower corticosterone than WT but after acute stressor significantly higher. Chronic 10mg/kg melatonin normalised serum corticosterone levels (not seen with vehicle or tianepetine)

*= Information obtained from contact with author. -=data not available. /=not tested. w=weeks. d= days, m= minutes. h= hours. EPM= elevated plus maze. ns=non-significant

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Table 2

Participant characteristics in studies investigating cortisol secretion in humans with Fragile X Syndrome

Study	FXS participants			Control Participants		
	N (M/F)	Age	Number with Autism	N (M/F)	Age	Characteristics
Bricout et al (2008)	1 M	24y	N/A	15 (M)	-	"Healthy"
Hessl et al (2002)	39 (F), 70 (M)*	6-17y (mean: 10.8y)	N/A	58 (F) 51 (M)	6-17y (mean 11.26)	Unaffected siblings. Confirmed absence of FXS or pre-mutation using southern blot.
Hessl, Glaser, Dyer-Friedman, & Reiss (2006)	32 (F) 58 (M)*	6-17y (Mean 10.89)	N/A	53 (F) 37(M)*	6-17y (mean 11.13)	Unaffected siblings. (Confirmed absence of FXS or pre-mutation using southern blot.)
Hall, DeBernadis & Reiss (2006)	40 (F) 74 (M)*	6-17y (male mean:11.06y, female mean 10.42y)	N/A	-	-	-
Hall, Lightbody & Reiss (2008)	29 (F) 31 (M)	5-20y (M mean: 13.21, F mean, 13.06)	16 M and 6 F autism (23 M and 13 F autism spectrum)	-	-	-
Roberts et al (2009)	51 (M)	FXS-only- mean 3.99y; FXS+ASD± mean 3.55y	18 with autism	21 (M)	Mean: 4.05y	Gender-matched typically developing (TD). No test *FMR1*

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						status.
Scherr, Hahn, Hooper, Hatton & Roberts (2016)	31 (M)**	9.67-14.58y (Mean 12.4, SD 1.29)	N/A	49 (M)**	4.92-9.5y (Mean 7.0y, SD 1.04y)	TD, matched on non-verbal mental age at beginning of longitudinal study.
Wisbeck et al (2000)	7 (F) 8(M)	6-25y (M mean 13.5y, F mean 13.9y)	N/A	41 (F) 43 (M)	Mean 7.5y	Non-matched normative sample. Data analysed in same laboratory.

ns= non-significant. m=minutes. y=years M=male, F=female

*Note: same group of participants in three studies. ~~group of participants as Hessl and colleagues (2002)~~

**Sub-set of total study participants for whom cortisol data was available.

[+] Fragile X Syndrome and high levels of autism symptomatology (as indicated by a score on the Child Autism Rating Scale (CARS; Schopler, Reicher & Renner, 1988) above the cut-off for an autism spectrum disorder)

[-] Fragile X Syndrome and low levels of autism symptomatology (as indicated by a score below the cut-off for an autism spectrum disorder on the CARS)

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Table 3

Comparisons of cortisol levels between groups of individuals with Fragile X Syndrome or comparison groups

Study	Stressor	Cortisol test		Cortisol Findings	
		Method	Sample Timings	~~Within group (FXS)~~	~~Between groups (control comparison)~~Group comparisons
Bricout et al (2008)	Sub-maximal incremental physical exercise treadmill test	Blood (venous Catheter)	At rest (8.30am), start of test, exercise+10m, exercise+20m, exercise+40m, recovery+30m, recovery+60m.	FXS cortisol elevated during the first 20 minutes of the test (start inclusive) compared to controls and showed a decrease at exercise+40m, opposite to controls who showed an increase	

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Hessl et al (2002)	-	Saliva (Salivette roll soaked 1-2 m). No citrus <30m, no dairy <60m	*Evaluation day*. 30m after waking, during testing (11am), prior to social challenge (3.30pm), 30m after social challenge, 90m after social challenge, bedtime. Cortisol levels for each sample were standardised by z-score transformation and averaged across the evaluation day to create composite score. *2 consecutive typical non-school days*. Within 30m waking, before breakfast, one hour before lunch, one hour prior to dinner, bedtime. Cortisol levels for each sample were standardised by z-score transformation and averaged across the typical days to create composite score.	~~*Typical Day.* Males and females both exhibited a normal diurnal decline. Males showed slower decline (higher cortisol) post-lunch until bedtime than females.~~ ~~*Experimental Day.* Males had greater response to visit: less decline (higher levels) between pre-breakfast and pre-lunch. Possibly related to meeting novel experimenter.~~ *Typical Day.* Male FXS cortisol elevated compared to siblings on typical days (as indicated by reduced diurnal decline) but not females. *Experimental day.* Females did not differ from siblings. Males showed higher levels between pre-breakfast and pre-lunch samples.
Hessl, Glaser, Dyer-Friedman, & Reiss (2006)	Social Challenge (in home) modified from protocol used by Herbert, Bellack and Hope (1991).	Saliva (Salivette cotton roll soaked 1-2 m). No citrus <30m, no dairy <60m	2 samples: prior to social challenge (~3pm) and 30m after beginning social challenge	~~No gender differences in FXS participants.~~ FXS showed higher pre-challenge levels than siblings. No differences in degree of change or post-challenge levels. FXS participants showed

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	Counterbalanced presentation of one 15-20m session of including the following conditions: child interview, silent reading, oral reading, singing.			increased cortisol through whole home assessment period (reported in Hessl et al 2002)	
Hall, DeBernadis & Reiss (2006)	Social Challenge. Conducted in-home at approximately 3pm. Fixed order presentation of one 15-20m session of each of the following conditions: child interview, silent reading, oral reading, singing.	Saliva (Salivette cotton roll 1-2 m)	One pre-challenge sample 3pm	-	Formatted: Font: Cambria Formatted: Font: Cambria
Hall, Lightbody & Reiss (2008)	In home assessment including intelligence and autism testing.	Saliva (Salivette cotton roll 1-2 m)	Evaluation day pre-breakfast (8am), pre-ADOS-G (3pm), pre-dinner (5pm), and pre-bedtime (9pm).	~~No main effect of gender~~	Formatted: Font: Cambria Formatted: Font: Cambria Formatted: Font: Cambria
Roberts et al (2009)	Naturalistic interactions with experimenter	Saliva (Salivette cotton roll soaked 1-2 m). No citrus or dairy <60m	Pre-assessment and post-social approach assessment.Time of day not specified.	FXS+ASD higher baseline and post-assessment than FXS-only. No group difference in magnitude of response. FXS+ASD higher post assessment and baseline than TD. No differences FXS-only and TD. No differences in magnitude of response. ~~FXS+ASD higher post assessment and baseline than TD. No differences FXS-only and TD. No~~	Formatted: Font: Cambria Formatted: Font: Cambria

				~~differences in magnitude of response.~~	
Scherr et al (2016)	Neurocognitive assessment battery	Saliva (Salivette, 1-2 m)	Baseline 15m (pre-assessment: 9am) and conclusion of assessment (12pm). Taken in Year 1, 2 and 3 of longitudinal assessment	Visual trend for increase in baseline cortisol over time (each year of longitudinal study). Not seen in TD. Both groups showed lower reactant cortisol than baseline. Year 1: FXS had significantly higher reactant than TD. Not significant at other time points. Non-significant trend for FXS to show greater change in time of cortisol (reactant-baseline) than TD. ~~Both groups showed lower reactant cortisol than baseline. Year 1: FXS had significantly higher reactant than TD. Not significant at other time points. Non-significant trend for FXS to show greater change in time of cortisol (reactant-baseline) than TD.~~
Wisbeck et al (2000)	Social Challenge modified from Herbert and colleagues' protocol (1991). Two 2-minute interpersonal role-play tasks: speech/song and reading aloud.	Saliva (Salivette cotton roll soaked 1-2 m). No citrus <30m or dairy <60m	*Day 1: evaluation day.* Pre-breakfast, 30m post-stress, 90m post-stress, pre-dinner, bedtime. *Days 2&3: routine days*. Pre-breakfast, pre-lunch, pre-dinner (no data for normative sample), bedtime. Average taken at each time-point across 2 days.	*Routine Days.* Compared to normative, FXS higher at lunch and bedtime (no pre-dinner sample to compare) ~~*Routine Days.* No male and female difference.~~ ~~*Experimental Day.* Males significantly higher than females 30m post-stressor and before bedtime.~~ ~~*Routine Days.* Compared to normative, FXS higher at lunch and bedtime (no pre-dinner sample to compare).~~

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Table 4

Gender comparisons of cortisol levels in individuals with Fragile X Syndrome

Study	Participant Type	N	Aspect of HPA activity measured	Gender comparison findings
Hessl et al (2002)	Human	39 (F), 70 (M)	Typical day circadian rhythm (average 2 days)	Males and females both exhibited a normal diurnal decline. Males showed slower decline (higher cortisol) post-lunch until bedtime than females.
			Experimental day circadian rhythm (involves novelty and social challenges)	Males had greater response to visit than females: less decline (higher levels) between pre-breakfast and pre-lunch. Possibly related to meeting novel experimenter.
Hessl, Glaser, Dyer-Friedman, & Reiss (2006)	Human	32 (F) 58 (M)*	Reaction to social challenge (pre and post measures)	No gender differences in FXS participants.
Hall, Lightbody & Reiss (2008)	Human	29 (F) 31 (M)	Collection at four time points during evaluation day	No main effect of gender
Wisbeck et al (2000)	Human	7 (F) 8(M)	Typical day Circadean rhythm (average 2 days)	No male and female difference.

			Experimental day circadian rhythm (involves novelty and social challenges)	Males significantly higher than females 30m post-stressor and before bedtime.
Markham et al (2006)	Mouse	8-12 per group	Response to acute stressor (restraint)	Different patterns of response and recovery to 30m of restraint stress. Males show protracted return to unstressed baseline; females show protracted rise. Peak secretion does not differ.

Table 54

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Studies assessing associations between cortisol and behaviour in individuals with Fragile X Syndrome

Study	Behavioural measure			Association of behaviour with cortisol?			
	Topic	Method	Typical Day	Experimental Day			Other
				Pre-challenge	Reactivity	Post-challenge	
Hessl et al (2002)	Problem Behaviour	Child behaviour checklist (CBCL; Achenbach, 1991). Total and sub-scale scores. Controlled for other factors associated with behaviour problems (See full text)	*Female.* Typical day composite significantly positively correlated with attention problems.	-	-	-	*Males.* Composite cortisol level (unspecified) accounted for 8% of variance in total behaviour problems. Higher levels were associated with increased behaviour problems, especially withdrawn behaviour. *Female.* Cortisol levels account for 14% of variance in behaviour problems. Evaluation composite significantly positively correlated with

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								social and attention problems.
Hessl et al (2006)	Social Escape	Measurement of gaze, vocal quality, discomfort and non-verbal task avoidance during social challenge.	-	-	Higher cortisol reactivity controlling for pre-challenge levels) associated with more gaze avoidance in siblings but opposite pattern in FXS (blunted response associated with increased gaze avoidance) for both males and females. No other associations found.	-	-	
	Problem Behaviour	Aberrant behaviour checklist (ABC; Aman et al, 1985); CBCL and Autism Behaviour Checklist (Krug, Arick, & Almond, 1993)	-	-	Increased cortisol reactivity associated with increased sensory and social relation problems in FXS (no other associations). No associations in sibling group.	-	-	

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Hall et al (2006)	Social escape	Measurement of gaze, refusals, face-hiding, eye-rubbing, hand-biting, fidgeting, leaving chair during social challenge.	-	In males, increased cortisol associated with decreased eye contact and increased fidgeting. No association with other social escape behaviours or number of problem behaviours seen.	-	-	-
Hall et al (2008)	Autistic Behaviour	Autism Diagnostic Observation Schedule-General (ADOS-G; Lord, Rutter, DiLavore, & Risi, 2002).		In males only, more autistic behaviour associated with lower cortisol.	-	-	-
	Compulsions	Compulsive Behaviour Checklist (Bodfish, Crawford,Powell & Parker, , 1995)	-	No association cortisol and prevalence of compulsions.	-	-	-

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	Self-Injurious Behaviour (SIB)	Self-injury checklist (SIB-C; Bodfish et al, 1995)	-	No association between cortisol and prevalence or number of forms of SIB.	-	-	-
Roberts et al (2009)	Autistic Behaviour (AB)	Scores on Childhood Autism Rating Scale (CARS; Schopler, Reichler, & Renner, 1986)	-	No associations	Decreased cortisol change associated with increased autistic behaviour in FXS+ASD (only)	No associations	-
	Social approach	Social Approach Scale- modified (Goldsmith & Lemery, 2000; Roberts et al, 2007): Initial and familiar approach (physical movement, facial expression & eye contact)	-	No associations in FXS group. In TD group increased cortisol associated with increased facial and eye contact during familiar social approach.	No association in FXS group. In TD group increased cortisol change associated with increased facial and eye initial social approach (no other associations)	FXS+ASD higher post-challenge cortisol associated with decreased initial physical approach.	-
Scherr et al (2016)	Verbal working memory	Score on Memory for Words Sub-test of *Woodcock–Johnson Tests of Cognitive Abilities, Third Edition* (WJ-III, Woodcock,	-	Higher baseline cortisol was associated with poorer performance on memory for words working	No significant association.	No significant association.	-

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		McGrew, & Mather, 2001).	memory test, for both groups.		
	Verbal working memory	Auditory working memory sub-test of *Woodcock–Johnson Tests of Cognitive Abilities, Third Edition* (WJ-III, Woodcock, McGrew, & Mather, 2001).	Increased baseline cortisol associated with decreased performance in the FXS group, only.	No significant association.	Overall fixed effects for auditory working memory and cortisol change was significant, there were no significant effects of cortisol change or group.

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Hypothalamic-Pituitary-Adrenal Axis Function in Fragile X Syndrome and its Relationship to Behaviour: A Systematic Review

Introduction

Fragile X Syndrome (FXS) is the most common known cause of inherited intellectual disability and the leading monogenetic cause of autism (Muhle, Trentacoste & Rapin, 2004; Turner, Webb, Wake, & Robinson, 1996), affecting approximately 1:4000 males and 1:8000 females (Sherman, 2002). Verkerk and colleagues (1991) categorized the genetic locus of the disorder as being an expanded CGG repeat on the long arm of the X chromosome, in the 5' untranslated region of the *FMR1* gene, occurring during maternal transmission. An expansion of 200 or more repeats typically causes the *FMR1* gene to become abnormally hypermethylated, silencing the production of the Fragile X Mental Retardation Protein (FMRP; Fu et al, 1991). FMRP is a ubiquitous transporter protein which carries target messenger ribonucleic acids (mRNAs; which contain genetic information) from the cell nuclei to ribosomes, where the information is decoded to produce specific amino acid chains for protein synthesis ((Feng et al., 1997; Irwin, Galvez & Greenough, 2000; Khandjian, Corbin, Woerly & Rouseau, 1996). The mRNAs served by FRMP have a broad range of purposes, though are largely involved in dendritic structure and function (Feng et al., 1997; Weiler et al., 1997).

The severity of the manifestation of the syndrome is variable, but individuals with FXS typically show marked behavioural features including deficits in attention, language and IQ; hyperactivity; anxiety; self-injury (particularly hand-biting); aggression; hyperarousal; stereotypies; and social difficulties, including gaze avoidance (Symons, Byiers, Raspa, Bishop & Bailey, 2010; Lachiewicz, et al., 1994; Bailey, Raspa, Olmsted & Holiday, 2008). The presentation of the FXS is quantitatively gender

dimorphic, due to the X-linked nature of the syndrome, with males typically (though not always) being more clearly affected than females. Crucially, anxiety plays a central role in many of the characteristic behaviours of the syndrome, including avoidance and confrontational behaviours (Sullivan, Hooper & Hatton, 2007) as well as autistic-like behaviour (Talisa, Boyle, Crafa & Kaufmann, 2014). It has long been hypothesised that aberrant or exaggerated physiological arousal, particularly stimulus-bound, may underlie these traits. Due to these tendencies for individuals with the condition to exhibit exaggerated behavioural responses to stressors, researchers have begun to investigate arousal and stress-related circuits and their relevance for individuals with FXS.

The hypothalamic-pituitary adrenal (HPA) axis is one of the body's main stress effector systems and is a circuit of interest in Fragile X research. Activation of the HPA axis triggers the release of glucocorticoids (such as cortisol in humans), which can be measured through blood or saliva sampling (Jessop & Turner-Cobb, 2008). Baseline release of cortisol follows a pronounced circadian rhythm marked by a peak after awakening, followed by a gradual decrease through the day, reaching a quiescent period during sleep (van Cauter, 1990): corresponding to the rest-activity cycle. Superimposed on this pattern, in response to physical or psychological stressors, is further pulsatile release of glucocorticoids, which is a normal, adaptive component of coping (Gunnar, 1987). In the short term the physiological changes associated with this are adaptive in that they help to provide resources for successful coping, though enduringly high cortisol levels may have harmful effects (McEwen, 1998). Multiple negative feedback loops in the HPA axis exist to maintain adaptive levels of cortisol (Herman & Cullinan, 1997). In addition, when stressors are chronic, the HPA system may reach a stage of

exhaustion (Selye, 1956) resulting in blunted cortisol levels and responses or even development of a pattern of decreases in response to stressors as a result of habituation (Miller, Chen & Zhou, 2007; Grissom & Bhatnagar, 2009).

Individual differences in HPA activity may also be important correlates or modifiers of social behaviour and behavioural and psychological responding to stressors. For instance, it has been noted that individuals with Cushing's syndrome (a condition characterised by chronically elevated baseline levels of cortisol) are more likely to experience negative psychological states, such as depression and anxiety (Kelly, Kelly & Faragher, 2003), suggesting that HPA hyper-activation may modify affect. In addition, research into shyness with participants of various ages suggests a complex functional interplay between cortisol and the regulation of social behaviour (for instance: Schmidt et al, 1997; Beaton et al, 2013; Kagan et al, 1988). Furthermore, there is a growing body of literature suggesting that individuals on the autism spectrum (which is characterised by atypical social behaviour) experience stress-related cortisol responses of increased magnitude and/ or duration (for instance: Spratt et al, 2012; Corbett, Mendoza, Abdullah, Wegellin & Levine, 2006), which may be driven by impaired negative feedback (Hoshino et al, 1987). Given the close association between FXS and autism spectrum disorder (ASD, for instance: Bailey et al, 1998), it will be important to consider autism symptomatology in the interpretation of FXS research.

In light of these associations between cortisol and behaviour, it is interesting that researchers investigating the FMRP target mRNAs have discovered an association between FXS and the HPA axis. *FMR1* knockout mice (an animal model of FXS) have been found to have fewer glucocorticoid receptors (GR-α) within neuronal dendrites, which would decrease homeostatic feedback regarding levels of cortisol (Jafari, Seese,

Babayan, Gall & Lauterborn, 2012; Miyashiro et al, 2003). Furthermore, in human subjects with FXS (but not typically developing or intellectually disabled controls), Annexin 1, a phospholipid-binding protein which mediates the inhibition by glucocorticoids on the HPA axis (Jessop, 1999), was synthesised and expressed abnormally (Sun, Cohen and Kaufmann, 2001). The level of dysregulation was closely associated with participants' level of FMRP, suggesting a direct regulatory relationship. Thus, it appears that lack of FMRP may result in excessive activation of the HPA axis, through impairing the negative feedback loop (Hessl et al, 2002). This highlights a pathway whereby cortisol regulation may be altered in FXS, which could play a direct or indirect relationship in the manifestation of the behavioural phenotype. Interestingly, broader studies of endocrine function in FXS, such as atypical negative feedback regulation of the thyroid (Bregman, Leckman & Rot, 1990) and cases of precocious puberty (Butler & Najjar, 1988; Moore, Chudley & Winter, 1990; Kowalczyk et al, 1996), support the presence of disturbances of the function of the hypothalamus and/or pituitary, which highlights another avenue to atypical cortisol regulation in the syndrome (Hessl, Riviera & Reiss, 2004).

There are also further features of FXS which may have relevance for the function of the HPA axis, a number of which we will review. Firstly, brain changes related to FXS may influence the emotional evaluation of events. Activation of the stress-effector systems relies on the evaluation of a stimulus or event by the limbic system: the "emotional centre" of the brain. One of the key components of this system, the amygdala, appears to be changed in FXS (Suvrathan & Chattarji, 2011). Broader excitatory and inhibitory imbalances in the FXS brain may also influence responding, in particular, a key glutamate receptor (MGluR5) which is affected in FXS plays an

important role in fear memory formation in the amygdala (Bear, 1998; Rodrigues et al, 2002). In turn, functional neuroimaging (fMRI) research has highlighted resultant atypical fear-specific functioning of the amygdala and a possible association between these brain changes and socioemotional deficits in individuals with FXS (Kim et al, 2012). These emotional-evaluative changes may clearly have downstream implications for cortisol release. Additionally, the gamma amino butyric acid neurotransmitter system appears to be commonly disordered in many neurodevelopmental disorders, including FXS (Braat & Kooy, 2015). Preclinically, FMRP has been shown to regulate GABA-ergic synaptic vesicle dynamics within the hippocampus of the Fmr1 KO mouse model, (Broek et al., 2016). Such genetically induced changes in the relative tonus of excitatory / inhibitory neurotransmitters within key limbic brain structures could potentially predispose to changes in stress responding. Finally, circadian rhythmicity (in terms of behaviour and biological clock component mRNAs in the liver) has also been shown to be deficient in mice lacking FMRP (Zhang et al, 2008); this broader disturbance of the biological clock may affect the pattern of activity in the baseline secretion of glucocorticoids via the HPA axis. The previous discussion highlights multiple ways in which HPA activity (as expressed in glucocorticoid levels) may be altered in FXS. In turn, activity in this system may directly modify and/or be indirectly associated with clinically significant behaviours in the syndrome (Hessl et al, 2002). As a consequence, research into the secretion of glucocorticoids has begun to emerge within the FXS literature. The aim of this review is to collate findings relating to HPA functioning in animal models of and humans with FXS. The inclusion of preclinical literature has been made in order to be able to conduct an in-depth analysis of the potential relationship between FXS and HPA function. The review addresses several questions:

a) Do individuals or animals with FXS exhibit atypical levels of glucocorticoids at baseline, or differences in the duration or magnitude of responses to stressors, compared to controls?
b) Given the X-linked nature of the condition, are there gender differences in the difference aspects HPA activity, in FXS?
c) Do measures of HPA activity relate to behaviour, in individuals with FXS?

Method

Selection Criteria for Studies

Types of studies. We considered relevant empirical or observational studies, written in English, which assessed measures of HPA output (cortisol in humans or corticosterone in mice, collected via salivary or haematological methods) in humans with full-mutation FXS or an animal model of the human full-mutation, such as the *FMR1* knock-out (KO) mouse. Papers were included if they contained either a group comparison of corticosterone levels or an analysis investigating the relationship between HPA activity and behaviour in individuals or animals with FXS. Case studies were considered when the individual's results were compared to normative data or matched with an individual without FXS.

Search Methods for Identification of Studies

Electronic search. The following databases were searched: Web of Science, SCOPUS, PubMed, and Academic Search Complete. The search was completed in June 2016.

Search terms. The search terms used for the HPA axis search were: (("fragile x" OR *FMR1*) AND (glucocorticoid* OR cortisol OR corticosterone)). The fields 'title',

'abstract' and 'keywords' were searched (or closest available option within the database).

Searching other resources. Bibliographies of relevant articles were scrutinised. Furthermore, the titles of studies published in the following journals were searched, using the same terms, to ensure no papers had been missed in the database search: Psychoneuroendocrinology; American Journal of Medical Genetics; Journal of Intellectual Disability Research. These searches yielded no additional papers.

Search Results

The search is depicted in Figure 1. In total, 79 unique papers were identified in the initial search, of which 17 met the inclusion criteria for this systematic review.

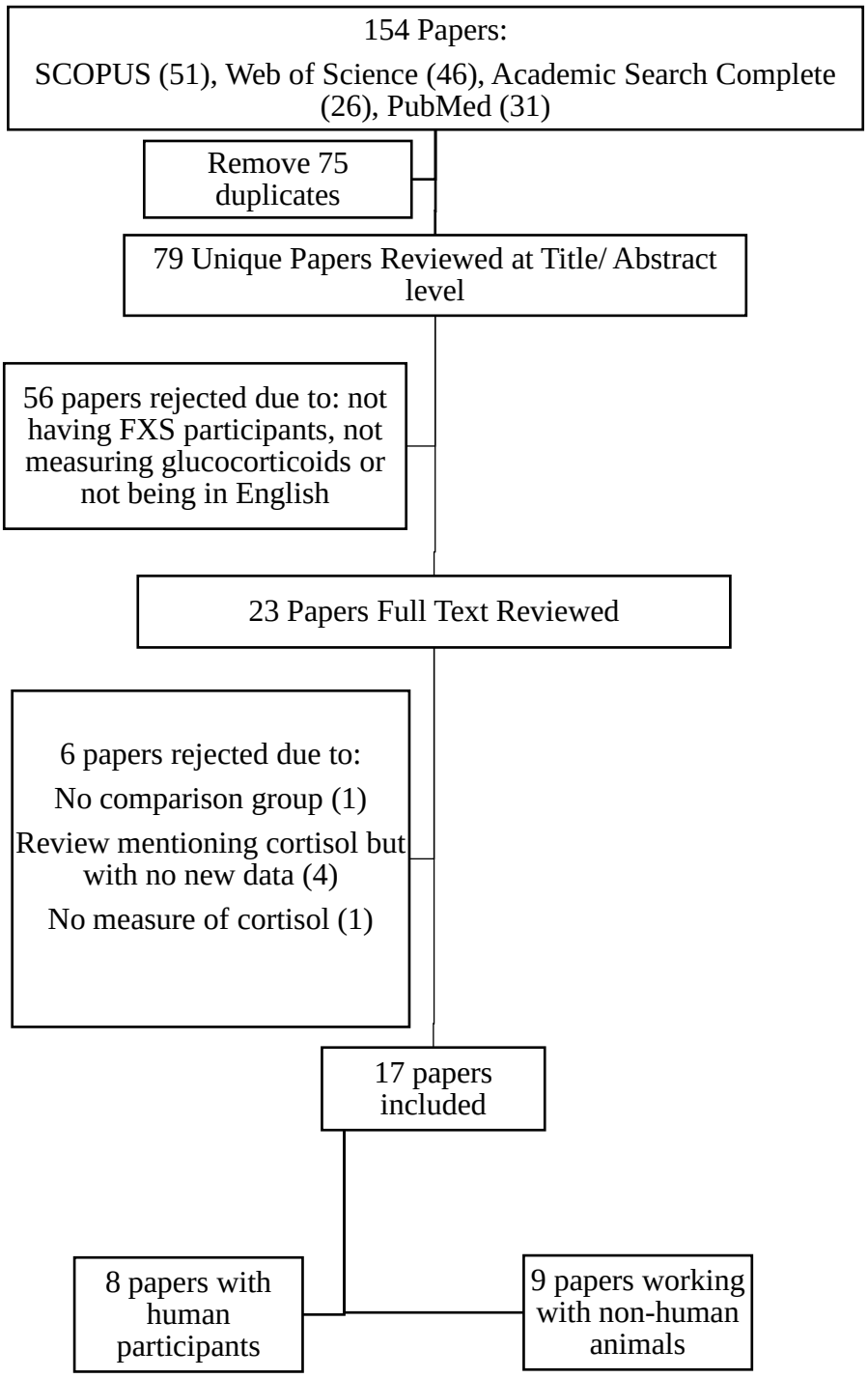

Figure 1. Depiction of the manuscript search process

Results and Discussion

Do Individuals or Animals with FXS Exhibit Atypical Levels of Glucocorticoids at Baseline, or Differences in the Duration or Magnitude of Responses to Stressors, Compared to Controls?

Baseline HPA activity and circadian rhythm.

Animal literature. Several studies (Table 1, includes summary of methodology and animal characteristics) have investigated the non-stressed corticosterone secretion of male KO mice compared to wild-type (WT) control animals, in order to identify whether changes exist in the baseline activity of the HPA axis in FXS animal models. The majority of studies found no genotype effect in their comparisons at single time-points, with male animals (Markham et al, 2006; Lauterborn, 2004; Nielsen et al, 2009; Eadie et al, 2009; Ghilan et al, 2015). Furthermore, in a more detailed analysis, Qin and Smith (2008) assessed the baseline circadian rhythm of both genotypes and found no difference at any of the six time-points tested (Qin & Smith, 2008). However, two studies did identify genotype differences, though the nature of the difference contrasted: de Diego-Otero and colleagues (2009) found that KO mice had lower corticosterone levels at baseline than WT controls; in contrast, Qin and colleagues (2011) found a main effect whereby KO mice generally had higher corticosterone than WT controls. As such, there is no evidence to suggest baseline HPA activity is altered in males with FXS, based on the preclinical evidence.

Gender differences. No studies of this nature have utilised female animals, as such it is unclear whether any gender differences exist in this area (gender comparisons summarised in Table 4).

Human Literature. Research investigating baseline HPA activity in humans has focussed upon profiling the diurnal rhythm of cortisol levels in this group (Table 3). Namely, two studies investigated cortisol levels through routine days (without unusual or exciting events). Wisbeck and colleagues (2000) conducted a pilot study involving 7 females and 8 males (between the ages of 6-25 years) with FXS, comparing to a normative sample, Hessl and colleagues (2002) later built upon this with a larger study of 39 females and 70 males with FXS (age 6-17 years) compared to siblings without FXS (58 female, 51 male; age 6-17 years). In both studies, boys with FXS exhibited higher levels of cortisol, resulting from reduced diurnal decline, than their unaffected siblings. These findings may be consistent with the hypothesis, from preclinical literature on mRNA targets, of disordered HPA negative feedback. However, the only way to separate the direct influences of HPA feedback regulation and the influence of broader differences originating from, for instance, atypical emotional evaluation of the environment, would be to directly challenge the HPA axis, for instance with a dexamethasone suppression test (as used by Hoshino and colleagues (1987) with individuals with autism). As mentioned previously, heterogeneity in the preclinical literature in terms of both methodology and results means that it is challenging to draw conclusions about any potential results between the findings in mice and these suggestive findings of blunting of circadian glucocorticoid release in humans with FXS. Further investigations of circadian rhythmicity in the HPA axis in *FMR1* KO mice may help to establish further evidence to understand these observed differences better.

Gender differences. Of note, there are suggestive gender-differences apparent in the observations. Though no differences were observed in the initial smaller study (Wisbeck et al, 2000), in a later study, with larger numbers of participants (Hessl et al, 2002), visual analysis of the data revealed that the cortisol profiles of the females with FXS closely corresponded with those of their unaffected siblings: whereas the males showed more pronounced differences in cortisol levels. Of note, however, the statistical significance of these differences were not evaluated (gender comparisons summarised in Table 4).

HPA reactivity to challenges. Early hypotheses suggested that stimulus-bound arousal differences (Cohen, 1995) may play a significant role in the behavioural phenotype of FXS. Evidence to evaluate this claim has been collected across a small number of studies, involving both human and non-human animal participants.

Animal Literature. Exposing animals to acute stress paradigms allows for investigation of the magnitude and/or duration of HPA axis reactions, and whether these differ in the *FMR1* KO model of FXS, compared to their WT counterparts. A commonly used trigger for acute stress with mice is to restrain the animal (for instance, in a small tube) for a period of time. A summary of this research is included in Table 1.

Magnitude of response. Seven studies were identified which had compared the magnitude of responses of male KO and WT mice to this procedure (implemented for between 15 minutes and three hours; Table 1). Three of these studies found that KO mice exhibited higher levels of corticosterone compared to WT controls, following the stressor. De Diego-Otero and colleagues (2008) observed this difference following 15 minutes of restraint stress. In contrast, Lauterborn (2004) found a significant difference in corticosterone responses only after more prolonged restraint (2 hours), however

only a trend towards a difference was observed with a shorter stressor (30 minutes). Ghilan and colleagues (2015) observed higher corticosterone levels after a short period of restraint (15 minutes) in the KO mice, compared to the WT mice. However, following more prolonged periods of restraint (30 and 60 minutes) both KO and WT mice showed responses which did not significantly differ in magnitude. Increased stress-related elevations were also seen in response to a different stressor (spatial novelty) by Romero-Zerbo and colleagues (2009), who found that, despite initially lower baseline corticosterone levels in the KO mice, following stressors the KO mice exhibited higher levels of corticosterone than their WT counterparts.

In contrast to the four studies finding elevations in KO mice responses, Eadie and colleagues (2009) found that KO mice had significantly lower corticosterone than WT, following 3 hours of restraint stress, suggesting a smaller hormonal response to the paradigm. Furthermore, there were no genotype differences observed in seven studies: three studies did not observe any genotype difference in the magnitude of corticosterone responses to restraint stress (Markham et al, 2006; Nielsen et al, 2009; Qin & Smith, 2008) and a further four studies also observed no difference using other acute stress paradigms, including exposure to spatial novelty (Nielsen et al, 2009; Qin & Smith, 2008; Qin et al, 2011) and swim stress (Nielsen et al, 2009).

Of interest, given the atypical social profile associated with Fragile X, de Diego-Otero and colleagues (2008) investigated the mice's reactions to both physical (restraint) and social stressors (housing with between 9 and 11 other animals for 15 minutes), to investigate whether there may be differences in the nature of corticosterone responses. They found that KO mice showed lower levels of corticosterone following the social stressor than WT mice, which differs from the trend

for elevations in response to restraint stress in other studies. This preliminarily suggests that the nature of the stressor (social verses physical) may be of importance when investigating stress-related physiology in the FXS mouse model. Finally, another interesting manipulation was included in a study by Qin and colleagues (2011) who exposed both WT and KO animals to chronic restraint stress, before exposure to an acute stressor in the form of a novel environment (Qin et al, 2011). However, no interaction was found between the genotype and chronic stress, on the corticosterone responses.

In summary, given the high numbers of null findings no firm conclusions can be drawn about the magnitude of responses in FXS mouse models. Where differences were observed, however, the trend was for male animals to exhibit higher levels of corticosterone. A possible reason for this variation in results between studies may be related to the genetic background of the mice used. Mouse strain differences have been previously found to influence both the magnitude and duration of corticosterone responses to stressors (Shanks, Griffiths, Zalcman, Zacharko & Anisman, 1990) and have been hypothesised to be associated with conflicting results more broadly, when using the *FMR1* KO (Pietropaulo, Guilleminot, Martin, Amato & Crusio, 2011). Interestingly, Markham and colleagues (2006) found male KO mice had protracted responses to 30 minutes of restraint when compared to WT mice, using mice of a C57/Bl6 background; however Qin and Smith (2008) did not find any genotype differences after the same stressor when using FVB/NJ male mice. However, clearly, there may have been other methodological differences between the studies which caused the differences in the results (see Table 1 for summary of key study methodology). For instance, the timings of the testing of the animals (when specified) varied between 7am and 2pm. The active

phase of mice is typically during the night time, inverse to humans, with a peak at approximately 8pm (Gong et al, 2015), though of course housing and lighting conditions may cause this to vary. The time widows for testing across the reviewed studies overlapped substantially making comparisons challenging. However, this possible influence should be considered in future research and there is a need to establish better evidence on the link between sample timings, circadian rhythmicity and stress-related corticosterone release in *FMR1* KO mice, in order to facilitate the interpretation of the literature.

Duration of response. Next, several studies have investigated the duration of corticosterone responses. This was achieved by conducting timecourse studies involving sacrificing groups of mice at differing lengths of time following a restraint stressor. Interestingly, Markham and colleagues (2006) observed that the male KO animals showed a slower return to unstressed baseline than WT; a pattern which is consistent with the prediction of reduced HPA negative feedback. Though, two other studies did not find any genotype differences between male animals in response duration (Nielsen et al, 2009; Qin & Smith, 2008).

Gender differences. One study was identified which included female mice (Markham et al, 2006; gender comparisons summarised in Table 4). In this research, no difference was found in peak responding between male and female KO mice, following physical restraint for 30 minutes. However, female KO mice showed a different pattern of response and recovery to their male counterparts, but also atypical compared to the WT mice: the female KO mice appeared to show a protracted rise, as their the peak corticosterone level was at the final 60m sample, where levels would be expected to be falling (there were no gender differences in the WT animals). This suggests that animal

gender may play an important role in the outcomes of research into HPA output in FXS. However, further research is needed.

Human literature. Four studies to date have investigated group differences in the release of cortisol in response to cognitive, behavioural or physical testing (see Table 2 for details of study participant characteristics and Table 3 for details of between-group comparisons). Preliminary evidence for atypical regulation is provided by a case study of an adult male (age 24 years) with FXS who showed an atypical pattern of adaptation in response to physical exercise: an early increase in cortisol followed by a large decrease, opposite to the pattern of adaptation seen in the healthy controls (15 males; Bricout et al, 2008). Larger studies have also evaluated differences in the magnitude of cortisol reactions, focussing particularly on the response to social stressors (due to the atypical social behaviour associated with the syndrome). The findings of these studies are mixed. Firstly, Hessl and colleagues (2002) observed that males with FXS (70, age 6-17 years) showed reduced diurnal decline in the period after meeting unfamiliar researchers, compared to the siblings (58 female, 51 male, age 6-17 years), which the authors suggested may have resulted from an increased response to this social challenge. In addition, Scherr and colleagues (2016) found that, in the first year of the longitudinal study, boys with FXS (31, age 9-14 years) showed higher levels of reactant cortisol following an assessment battery, when compared to TD controls (49, matched on non-verbal mental age, 4-9 years). These differences were not observed in the following two assessment years, in which fewer individuals participated (Scherr, 2013). In addition, levels of baseline cortisol were higher in the FXS group than the comparison group, though this difference did not reach a level of statistical significance. In addition, the authors noted differences in the changes of cortisol levels over the

longitudinal assessment. Firstly, the degree of change in cortisol levels over the years of the longitudinal assessment (reactant minus baseline levels) increased in the FXS group, as compared to the TD controls. Visual analysis suggested that the baseline levels of cortisol increased over the years of assessment in the FXS group, but not the TD group. As such, the evidence from these two studies, as well as the aforementioned case study, suggest possible differences in the responses of boys with FXS, as well as differences in the development of this regulation over time.

However group differences were not observed in all studies. Further analysis of the data collected in the study by Hessl and colleagues (2002), did not find any differences between the children with FXS and unaffected siblings in cortisol levels in response to, or following, a structured social challenge (Hessl et al, 2006. FXS group: 58 males, 32 females, age 6-17 years. Sibling group: 53 females, 37 males, age 6-17 years). Finally, Roberts and colleagues (2009) conducted an evaluation of 51 males with FXS (mean age 3 years) compared to 21 male TD controls (mean age 4 years) investigated the magnitude of cortisol responses to a social interaction between children with FXS and their siblings without FXS, though divided the FXS group according to degree of autism symptomatology in their analysis. It was found that, although there were no differences between young boys with FXS and low levels of autism symptomatology (who did not meet the criteria for a dual diagnosis of autism spectrum disorder on the CARS) and their siblings, children with FXS and high levels of autism symptomatology had higher levels of cortisol both prior to and following social interactions with an unfamiliar experimenter (though there were no differences in the magnitude of the response). This suggests that there may be differences in cortisol profiles within the population of people with FXS, relating to the degree of autistic symptomatology. The

relationship between cortisol and autism symptomatology is discussed in further detail later in this review.

Thus, as with the findings in the preclinical literature, the findings of the studies in humans are heterogeneous. However, where differences were observed between the 'typical' or baseline cortisol levels of individuals with and without FXS, they were manifested as relative increases, rather than decreases, in cortisol secretion. This corresponds to the preclinical observations of comparatively higher corticosterone responses to stressors in *FMR1* KO mice in four studies; though, as mentioned above, seven studies found no genotype difference in these animals. However, this potential trend in the findings highlights an avenue for future investigation.

Gender differences. Given the broad gender differences in the manifestation of FXS, researchers have chosen to investigate whether there are differences in cortisol responses between males and females with FXS, in four studies. In two studies, it was observed that males showed higher levels of cortisol following social challenges (a brief social stressor: Wisbeck et al, 2000; interaction with an unfamiliar experimenter: Hessl et al, 2002) than females. This suggests that atypical responding may be limited to, or at least exaggerated, in males with FXS, compared to females with the condition, mirroring the observations in the preclinical literature on the topic. Of note, however, the differences in both studies were based on visual observances and were not statistically evaluated. In contrast, both Hessl and colleagues (2006) and Hall and colleagues (2008: 29 females and 31 males, age 5-20 years) found no gender differences in their studies, where statistical comparisons were conducted. Namely, Hessl and colleagues (2006) found no differences in the magnitude of response to a social challenge and Hall and

colleagues (2008) saw no differences in diurnal decline across a day which involved unfamiliar social interactions in the form of evaluations by the experimenters.

Therefore, the results between studies are mixed, which may, in part, reflect the higher variability in the presentation of FXS in females, resulting from processes such as X-inactivation. Though, in the wider literature, there is evidence of gender-related differences in HPA in adulthood, though it is unclear whether robust differences exist in younger individuals (Jessop & Turner-Cobb, 2008), such as those included in the studies in FXS. It is possible that there are also FXS-independent differences which contribute to this gender dimorphism. More detailed exploration of the relationship between other biomarkers (such as FMRP), cortisol and behaviour in males and females with FXS may help to clarify the origins of this variability and verify whether differences do exist.

Is there a Relationship Between Cortisol Levels and Behaviour within Fragile X Syndrome?

Animal literature. To date, there has been no research to date investigating whether individual differences in corticosterone responses relate to differences in behaviour. Though, it is unclear how such research may translate to understanding of human behaviour as the behavioural phenotype of the mouse model does not correspond closely to that of the human phenotype. Namely, in comparison to the increased levels of anxiety associated with FXS in humans (for instance; Cordiero et al, 2011), all studies which utilised a behavioural assay to assess anxiety (Elevated Plus Maze; Pellow, Chopin, File & Briley, 1985) observed that *FMR1* KO mice exhibit decreased behavioural indicators of anxiety relative to their WT counterparts (Qin et al, 2011; de Diego-Otero et al, 2008; Qin & Smith, 2008; Eadie et al, 2009). No research has been conducted to identify which mouse behaviours correspond to clinically significant

behaviours in FXS. However, should these be identified, animal models may help to highlight relationships between HPA axis function and behaviour in FXS.

Human literature. Five studies conducted within-group comparisons to investigate the relationship between salivary cortisol and measures of behaviour in individuals with FXS (see Table 2 for participant details and Table 5 for study details).

Social and Autistic Behaviours. Many people with FXS display autistic-like characteristics including: gaze-avoidance, repetitive behaviour and shyness. However, not all individuals with FXS display levels of autistic symptomatology which mean that they meet the diagnostic criteria for an autism spectrum disorder (Talisa et al, 2014). As such, a number of studies have investigated possible factors associated with the degree of autism symptomatology, including salivary cortisol.

Three studies which have utilised observational measures of behaviours exhibited by individuals with FXS, during various types of social interaction. Two of these studies, utilising the same group of participants, observed the behaviour of individuals with FXS during a structured social challenge, which involved asking the child to read, answer questions and sing in front of others (Hall et al, 2006; Hessl et al, 2006). Many of the measured behaviours were not found to have relationships with cortisol levels including: vocal quality (including mumbling or intrusive tones: Hessl et al, 2006) discomfort (participant appears in crisis, demonstrating behaviours such as self-injury, crying, aggression: Hessl et al, 2006. Hand-biting was also assessed separately in: Hall et al, 2006) non-verbal task avoidance (physically leaving the situation or covering eyes; Hessl et al, 2006; Hall et al, 2006), verbal refusals (Hall et al, 2006). Though, a positive correlation was observed with fidgeting (Hall et al 2006). Most interestingly, however, gaze avoidance, one of the characteristic features of the

FXS phenotype, was found to relate to levels of cortisol in both studies, though the direction of the associations differed. Hessl and colleagues (2006) found that (across males and females with FXS), after controlling for other potential influences on cortisol levels, increased gaze aversion was associated with a lower post-challenge levels, to the social challenge. In fact, it was noted that the most gaze aversive children exhibited decreases in eye contact in response to the challenge. In contrast, Hall and colleagues (2006) found that *increased* mean levels of cortisol were associated with decreased eye contact. However, these findings raise two hypotheses as to whether the primary influence on gaze avoidance relates to autistic-like characteristics (i.e. a lack of response to social stimuli) or social anxiety (i.e. an excessive response to social stimuli; Hessl et al; 2006). Both hypotheses are interesting and warrant further investigation.

Furthermore, the relationship between salivary cortisol and social approach behaviour during naturalistic social interactions has been explored in one study (Roberts et al, 2009). The method involved investigating social approach behaviour (physical approach, facial expressions and eye contact) with an experimenter when they were both unfamiliar (first minute of interaction) and familiar (during last hour of day-long assessment) to the child. Typically, as also seen in the controls in this study, children who approach an unfamiliar person more show an increased reaction and those who later approach the experimenter more (when familiar) initially had higher baseline levels of cortisol. However, the children with FXS showed a different pattern of association in this study. Firstly, the participants with FXS and low levels of autism symptomatology showed no significant association between cortisol and behaviour at all. Whereas, within the group of children with FXS and high levels of autism symptomatology, boys with higher cortisol levels (following the interaction) showed

fewer physical approaches to the unfamiliar experimenter: the opposite pattern to in the control group. As such, this study suggests a possible association between heightened physiological reactions to social situations, and increased social avoidance.

Further evidence on the association between cortisol and autistic behaviour in FXS comes from studies which have utilised broader autism screening or diagnostic measures. Hall and colleagues (2008) utilised a direct observational assessment measure (ADOS-G; Lord, Rutter, DiLavore, & Risi, 2002) with their participants. The results of the study indicated that lower baseline levels of cortisol were associated with higher levels of autistic behaviour. Hessl and colleagues (2006) also found a relationship between cortisol and some types of autistic behaviour: increased sensory and social relation problems were positively associated with cortisol reactivity to a social challenge. Roberts and colleagues (2009), in contrast, found that *reduced* cortisol reactivity (which the authors suggest could be related to elevated basal levels) to a social interaction was associated with increased autistic behaviour (as measured on a behaviour rating scale: CARS; Schopler, Reicher & Renner, 1980), only within the group of individuals with FXS and high levels of autism symptomatology; in the group of children with FXS and low levels of autism symptomatology, there was no relationship between cortisol and levels of autistic behaviour.

As such, a number of studies highlight associations between HPA activity and this key part of the FXS behavioural phenotype. However, the nature and direction of this association varies, with some finding increased levels of cortisol to be associated with increased autism symptomatology, both other with decreased cortisol levels. The heterogeneity of measures behaviour (direct observation as compared to informant rating scales) and cortisol may underlie such differences. In addition, the findings of

Roberts and colleagues (2009) raise the possibility that levels of cortisol more strongly relate to behaviour in individuals with FXS and high autism symptomatology, as compared to those with lower symptomatology. In fact, the authors suggest that HPA dysregulation may serve as a biomarker of ASD in FXS. This highlights that individuals' levels of autistic behaviour may be important to consider when interpreting the results of studies of the relationship between cortisol and behaviour in FXS. Though, variations in the assessment of autistic behaviour across the other reviewed studies make it challenging to evaluate this further based on the existing evidence. Future research to study the gradation of ASD in FXS would be valuable to delineate phenotypic boundaries and evaluate the significance of HPA function as a biomarker of ASD in FXS.

There is a growing body of literature relating to idiopathic autism which is also of relevance to this discussion. A review of this literature, revealed differences in both HPA rhythm and responsiveness in individuals with autism (Taylor & Corbett, 2014). Typically, in response to social situations, individuals with autism exhibit blunted responsiveness, which corresponds to the patterns seen by both Hall and colleagues (2008) and Hessl and colleagues (2006). Interestingly, however, the differences observed in those with idiopathic autism seem to be moderated by levels of functioning: there is not conclusive evidence that HPA dysregulation observed in lower functioning individuals also applies to individuals with high functioning autism. In Roberts and colleagues' study, the participants with both FXS and high levels of autism symptomatology had lower levels of adaptive behaviour than those with low levels of autism symptomatology (though the significance of the difference was not evaluated), highlighting a potential confound. Future research should examine this potential relationship in the FXS population.

A broader question relating to the question of the association between autistic behaviour in FXS is the debate as to whether autistic-like behaviours in individuals with FXS meaningfully correspond to the characteristics seen with idiopathic autism (Hall et al, 2010). It is possible that autistic-like behaviours in FXS have different causal mechanisms and, as such, the relationships between cortisol and behaviour may differ in those with syndromic and non-syndromic autistic characteristics. Future research might help to address these issues. For instance, comparison of the relationships between cortisol and behaviour in those with autism, including those with non-syndromic autism and those with FXS who meet the criteria for autism, may help to elucidate whether cortisol and behaviour relations differ in their nature or development, dependent upon genetic status.

Behaviour problems. Behavioural problems and challenging behaviours are a key issue of concern for many caregivers of people with FXS. Such behavioural issues are often anecdotally reported to be related to 'hyperarousal', meaning that objective investigations between these behaviours and physiological responses are warranted. In order to explore this issue, two studies have utilised the Child Behaviour Checklist (CBCL; Achenbach, 1991) as a broad measure of behaviour problems, and explored relations between scores and cortisol levels. Hessl and colleagues (2002; controlling for other factors which were found to be predictive of the scores) found that a composite score of cortisol significantly predicted 14% of the variance in total behaviour problem scores for females with FXS. Further analyses indicated that increased cortisol levels were specifically associated with increased social and attention problems. Furthermore, a composite score representing cortisol secretion on typical days significantly positively correlated with attention problems and approached significance for somatic complaints

and social problems. There were no other relationships between cortisol and other of the measured behaviour problems, including: withdrawn behaviour, anxious or depressed behaviour, thought problems, aggressive behaviour or delinquent behaviour. In the same study cortisol levels accounted for 8% of variance in total behaviour problems in the males with FXS, which approached significance. The strongest association with a subscale score was with withdrawn behaviour. In comparison, in a later study with the same participants (Hessl et al, 2006), no relationship was found between CBCL scores and any cortisol measures (baseline, post-challenge cortisol or magnitude of change) taken in relation to a social challenge.

Other Characteristics. Scherr and colleagues (2016) found that increased baseline levels of cortisol were associated with lower verbal working memory performance in boys with FXS, suggesting a possible link between arousal levels and academic-related performance.

Synthesis and Future Research

There are some interesting preliminary findings in this existing research. Though the findings are heterogeneous, there are some interesting observations and trends within the relatively few studies that have addressed the issue of HPA function in FXS, to date. In mice, no robust differences in baseline cortisol levels were seen, though there was some evidence of elevated stress-related reactivity. In human studies, baseline differences were observed in several studies (Hessl et al 2002; 2006; Roberts et al, 2009), as well as some indications of reactivity differences, compared to TD children (Scherr et al, 2016; Hessl et al, 2002; Roberts et al, 2009), though such differences may be mediated by gender and degree of autism symptomatology. At present, specific conclusions about the role of cortisol levels in behaviour associated with FXS are

difficult to draw due to the high levels of variability and lack of correspondence between studies. However, there are suggestions of associations between cortisol levels and autistic behaviour, behaviour problems and key cognitive processes (working memory). Future research will undoubtedly help to clarify some of these uncertainties and strengthen the evidence to clarify the robustness of the observed themes.

In addition to the suggestions discussed through the previous sections, there are several other considerations for future projects. The wider information on the HPA system highlights its complexity, with individual differences relating to multiple factors, including: medication, pubertal stage, gender, temperament, chronic stress, compliance with the sampling protocol, nature of stressors, familial genetics and BMI (Jessop & Turner-Cobb, 2008; Gunnar et al, 2003; Dickerson & Kemeny, 2004; Kupper et al, 2005). As well as the variation in study methodology, many of these potential influences have not been explored or accounted for in the research and may relate to the observed variability in study findings. Furthermore, the research to date has provided important but limited snapshots of the activity of the HPA axis, with the exception of Scherr and colleagues' (2016) longitudinal study, in small groups of individuals. Prospective or longitudinal studies including further information about an individual's characteristics, behaviour, environment and biology would help to provide a more detailed picture of the role of HPA activity in this population.

In addition, research involving humans has utilised TD comparison groups. However, an important step in future research will be to investigate the specificity of any group differences to individuals with FXS. It is possible that, rather than being directly FXS-related, the differences observed could relate to having an intellectual disability or autistic behaviour, and may be seen in others without FXS, but with similar

characteristics. For instance, earlier research with people with autism has highlighted that level of cognitive functioning relates to the findings: with differences being observed predominantly in those described as "low functioning" (Hoshino et al, 1987; Corbett et al, 2006; Richdate & Prior, 1992; Taylor & Corbett, 2014). In addition, given the aforementioned potential link also between levels of autism symptomatology and cortisol levels in those without FXS, and the high levels of autistic behaviour seen in people with FXS (Clifford et al, 2007), this clearly may be a confound in the differences seen. This is particularly pertinent given the findings of Roberts and colleagues (2009), who found that cortisol differences in FXS, compared to TD controls, was dependent upon levels of autism symptomatology. Thus, future research should employ control groups to help address these potential confounds, including those with non-syndromic autism and those with idiopathic intellectual disabilities.

In addition, in the human literature discussed above, much of the focus has been upon investigating HPA responses relating to social interactions or demands, in individuals with FXS. Given that individuals with FXS are prone to experiencing exaggerated behavioural responses, anxiety or phobias relating to many, varied situations (Cordeiro, Ballinger, Hagerman & Hessl, 2011), it is possible that idiosyncratic circumstances (outside of the examined social challenges or interactions) may also trigger cortisol responses that differ in magnitude or duration, compared to the general population. For instance, individuals with FXS are known to experience atypical sensory processing (Belser & Sudhalter, 1995) and have been shown to show elevated startle responses to sensory stimuli (Miller et al, 1999). Of note, research with individuals with autism has highlighted differential patterns of reactions to social-evaluative and non-social (such as unpleasant sensations) stimuli (Taylor & Corbett,

2014). Therefore, future research should address cortisol responses to a wider variety of situations which may be challenging for individuals with FXS, in order to gain a broader picture of HPA activity in this population, and it's potential applicability to day-to-day challenges.

Finally, given the possible significance of arousal differences, as evidenced by potential links with cognition and behaviour, researchers should also evaluate potential strategies for managing levels of arousal and systematically assess for any resultant improvements more widely. Scherr and colleagues (2016) highlight that individuals with FXS may benefit from targeted arousal-reducing interventions, such as the teaching of coping skills or relaxation techniques. This could include, for instance, the use of mindfulness-based techniques, which have been shown to reduce levels of arousal in individuals with Williams Syndrome (Miodrag et al., 2013). There has been a paucity of systematic evaluations of potential interventions for arousal issues in individuals with FXS, which include physiological measures, such as assessment of cortisol. However, Hall and colleagues (Hall, Lightbody, McCarthy, Parker, & Reiss, 2012) piloted intranasal oxytocin as an intervention for social anxiety in a small group of boys with FXS and found both increases in eye contact and decreases in cortisol levels after administration. This suggests that there may be avenues for both pharmacotherapy and behavioural interventions when seeking ways to support individuals with FXS in this area.

Conclusion

In summary, there is emerging evidence that cortisol levels differ in individuals with FXS compared to TD controls and relate to socially significant behaviours, thus highlighting a number of important avenues for future exploration. Delineating the

significance and role of HPA activity in the syndrome will help to further our understanding of the mechanisms of the condition and may lead to provision of more effective support.

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Table 1

Studies investigating corticosterone secretion in FMR1 knockout mice.

Study	Cort. measure	Method Sacrificed	Gender (M/F)	Mice per group	Strain	Age of Mice	Basal measure	Stress Condition(s)	Recovery Time	Time Tested	Cort. Findings
Ghilan et al (2015)	Blood serum from trunk	Decapitation following anaesthetisation by isoflurane	M	7-18	C57Bl/6	55-65d	/	Restraint (conditions: 15m/ 30m/ 1h) or control	None: quick sacrifice after restraint	9am-11am	WT mice showed significant elevations only after 30m or 1h of restraint. KO mice showed increases after all restraint periods. After 15m restraint, KO mice significantly higher corticosterone than WT. Suggests even short stress exposures trigger response in KO mice

de Diego-Otero et al (2008)	Blood plasma	Retro- orbital puncture	M	8-12	FVB-129	60-180d	/	Social stress (15m) or acute immobilisation stress (15m) or control	None: immediate sacrifice following behavioural . test battery	-	KO in control and social stress conditions lower corticosterone than WT. Acute stress KO higher corticosterone than WT.
Lauterborn (2004)	Blood plasma from right ventricle	Overdose with euthansol	M	-	FVB*	-	/	Restraint (30m/ 2h) control.	-	10am-2pm	Following 2h restraint KO higher corticosterone than WT, similar ns trend following 30m restraint
Markham et al (2006)	Blood serum from trunk	Rapid decapitation	M & F	8-12	C57/Bl6	40-45d	Cagemate sham comparisons (no restraint, just moved to test room)	Restraint (30m) or control	Conditions: 0/ 15/ 60m	10am - 12.30 pm	Male KO protracted return to unstressed baseline (still elevated at 60 m). Female show protracted rise compared to WT. peak secretion does not differ between

											genotypes.
Nielsen et al (2009)	Blood plasma from trunk	Rapid decapitation	M	5-12	FVB/NJ x C57/Bl6 (F1 hybrid)	11-12w	/	Swim Stress (3m) or open field (10m) or restraint (unspecified length). Each condition with control.	Swim: 17m. Open field: 10m. Restraint conditions: 0/ 30/ 60/ 90/ 120m	7am-9am	No genotype difference in magnitude or duration of corticosterone response to any stressor.
Qin, Xia, Huang & Smith (2011)	Blood plasma from trunk	Rapid decapitation	M	19-24	FVB/NJ	96±1d	/	Prior stress: chronic stress (2h/d restraint x10) or control. Acute stressor: spatial novelty (EPM)	-	-	No interaction between genotype and chronic stress condition. Main effect genotype: corticosterone higher in KO.
Qin & Smith (2008)	Blood plasma from trunk	Rapid decapitation	M	10-12	FVB/NJ	100±10 d	2am, 6am, 10am, 2pm, 6pm, 10pm	Acute restraint stress (30/ 120 m) or spatial novelty (EPM 5m) or control	Conditions: 30/ 120m	Before 11am	WT and KO no circadian rhythm differences (basal measures). Following stressors, no genotype difference in any

										condition
Eadie et al (2009)	Blood plasma from trunk.	Rapid decapitation	M	4	C57BL/6	-	Acute restraint stress (3 hours) or control	Immediate sacrifice following stressor	9am-1pm	No difference in control condition but following stressor NO showed significantly lower corticosterone.
Romero-Zerbo et al (2009)	Blood serum	Cervical dislocation	M	10-11	FVB-129	90-120d	Open field	Immediate sacrifice following stressor	-	At baseline, KO significantly lower corticosterone than WT but after acute stressor significantly higher. Chronic 10mg/kg melatonin normalised serum corticosterone levels (not seen with vehicle or tianepetine)

*= Information obtained from contact with author. -=data not available. /=not tested. w=weeks. d= days, m= minutes. h= hours. EPM= elevated plus maze. ns=non-significant

Table 2

Participant characteristics in studies investigating cortisol secretion in humans with Fragile X Syndrome

Study	FXS participants			Control Participants		
	N (M/F)	Age	Number with Autism	N (M/F)	Age	Characteristics
Bricout et al (2008)	1 M	24y	N/A	15 (M)	-	"Healthy"
Hessl et al (2002)	39 (F), 70 (M)*	6-17y (mean: 10.8y)	N/A	58 (F) 51 (M)	6-17y (mean 11.26)	Unaffected siblings. Confirmed absence of FXS or pre-mutation using southern blot.
Hessl, Glaser, Dyer-Friedman, & Reiss (2006)	32 (F) 58 (M)*	6-17y (Mean 10.89)	N/A	53 (F) 37(M)*	6-17y (mean 11.13)	Unaffected siblings. (Confirmed absence of FXS or pre-mutation using southern blot.)
Hall, DeBernadis & Reiss (2006)	40 (F) 74 (M)*	6-17y (male mean:11.06y, female mean 10.42y)	N/A	-	-	-
Hall, Lightbody & Reiss (2008)	29 (F) 31 (M)	5-20y (M mean: 13.21, F mean, 13.06)	16 M and 6 F autism (23 M and 13 F autism spectrum)	-	-	-
Roberts et al (2009)	51 (M)	FXS-only^{-} mean 3.99y; FXS+ASD^{+} mean 3.55y	18 with autism	21 (M)	Mean: 4.05y	Gender-matched typically developing (TD). No test *FMR1*

						status.
Scherr, Hahn, Hooper, Hatton & Roberts (2016)	31 (M)**	9.67-14.58y (Mean 12.4, SD 1.29)	N/A	49 (M)**	4.92-9.5y (Mean 7.0y, SD 1.04y)	TD, matched on non-verbal mental age at beginning of longitudinal study.
Wisbeck et al (2000)	7 (F) 8(M)	6-25y (M mean 13.5y, F mean 13.9y)	N/A	41 (F) 43 (M)	Mean 7.5y	Non-matched normative sample. Data analysed in same laboratory.

ns= non-significant. m=minutes. y=years M=male, F=female

*Note: same group of participants in three studies.

**Sub-set of total study participants for whom cortisol data was available.

[+] Fragile X Syndrome and high levels of autism symptomatology (as indicated by a score on the Child Autism Rating Scale (CARS; Schopler, Reicher & Renner, 1988) above the cut-off for an autism spectrum disorder)

[-]Fragile X Syndrome and low levels of autism symptomatology (as indicated by a score below the cut-off for an autism spectrum disorder on the CARS)

Table 3

Comparisons of cortisol levels between groups of individuals with Fragile X Syndrome or comparison groups

Study	Stressor	Cortisol test		Cortisol Findings
		Method	Sample Timings	Group comparisons
Bricout et al (2008)	Sub-maximal incremental physical exercise treadmill test	Blood (venous Catheter)	At rest (8.30am), start of test, exercise+10m, exercise+20m, exercise+40m, recovery+30m, recovery+60m.	FXS cortisol elevated during the first 20 minutes of the test (start inclusive) compared to controls and showed a decrease at exercise+40m, opposite to controls who showed an increase
Hessl et al (2002)	-	Saliva (Salivette roll soaked 1-2 m). No citrus <30m, no dairy <60m	*Evaluation day*. 30m after waking, during testing (11am), prior to social challenge (3.30pm), 30m after social challenge, 90m after social challenge, bedtime. Cortisol levels for each sample were standardised by z-score transformation and averaged across the evaluation day to create composite score.	- *Typical Day.* Male FXS cortisol elevated compared to siblings on typical days (as indicated by reduced diurnal decline) but not females. *Experimental day.* Females did not differ from siblings. Males showed higher levels between pre-breakfast and pre-lunch samples.

			2 consecutive typical non-school days. Within 30m waking, before breakfast, one hour before lunch, one hour prior to dinner, bedtime. Cortisol levels for each sample were standardised by z-score transformation and averaged across the typical days to create composite score.	
Hessl, Glaser, Dyer-Friedman, & Reiss (2006)	Social Challenge (in home) modified from protocol used by Herbert, Bellack and Hope (1991). Counterbalanced presentation of one 15-20m session of including the following conditions: child interview, silent reading, oral reading, singing.	Saliva (Salivette cotton roll soaked 1-2 m). No citrus <30m, no dairy <60m	2 samples: prior to social challenge (~3pm) and 30m after beginning social challenge	FXS showed higher pre-challenge levels than siblings. No differences in degree of change or post-challenge levels. FXS participants showed increased cortisol through whole home assessment period (reported in Hessl et al 2002)
Hall, DeBernadis & Reiss (2006)	Social Challenge. Conducted in-home at approximately 3pm. Fixed order presentation of one 15-20m session of each of the following conditions: child interview, silent	Saliva (Salivette cotton roll 1-2 m)	One pre-challenge sample 3pm	-

	reading, oral reading, singing.			
Hall, Lightbody & Reiss (2008)	In home assessment including intelligence and autism testing.	Saliva (Salivette cotton roll 1-2 m)	Evaluation day pre-breakfast (8am), pre-ADOS-G (3pm), pre-dinner (5pm), and pre-bedtime (9pm).	-
Roberts et al (2009)	Naturalistic interactions with experimenter	Saliva (Salivette cotton roll soaked 1-2 m). No citrus or dairy <60m	Pre-assessment and post-social approach assessment.Time of day not specified.	FXS+ASD higher baseline and post-assessment than FXS-only. No group difference in magnitude of response. FXS+ASD higher post assessment and baseline than TD. No differences FXS-only and TD. No differences in magnitude of response.
Scherr et al (2016)	Neurocognitive assessment battery	Saliva (Salivette, 1-2 m)	Baseline 15m (pre-assessment: 9am) and conclusion of assessment (12pm). Taken in Year 1, 2 and 3 of longitudinal assessment	Visual trend for increase in baseline cortisol over time (each year of longitudinal study). Not seen in TD. Both groups showed lower reactant cortisol than baseline. Year 1: FXS had significantly higher reactant than TD. Not significant at other time points. Non-significant trend for FXS to show greater change in time of cortisol (reactant-baseline) than TD.
Wisbeck et al (2000)	Social Challenge modified from Herbert and colleagues' protocol (1991). Two 2-minute interpersonal role-play tasks: speech/song and	Saliva (Salivette cotton roll soaked 1-2 m). No citrus <30m or dairy <60m	*Day 1: evaluation day.* Pre-breakfast, 30m post-stress, 90m post-stress, pre-dinner, bedtime. *Days 2&3: routine days*.	*Routine Days.* Compared to normative, FXS higher at lunch and bedtime (no pre-dinner sample to compare)

	reading aloud.	Pre-breakfast, pre-lunch, pre-dinner (no data for normative sample), bedtime. Average taken at each time-point across 2 days.	

Table 4

Gender comparisons of cortisol levels in individuals with Fragile X Syndrome

Study	Participant Type	N	Aspect of HPA activity measured	Gender comparison findings
Hessl et al (2002)	Human	39 (F), 70 (M)	Typical day circadian rhythm (average 2 days)	Males and females both exhibited a normal diurnal decline. Males showed slower decline (higher cortisol) post-lunch until bedtime than females.
			Experimental day circadian rhythm (involves novelty and social challenges)	Males had greater response to visit than females: less decline (higher levels) between pre-breakfast and pre-lunch. Possibly related to meeting novel experimenter.
Hessl, Glaser, Dyer-Friedman, & Reiss (2006)	Human	32 (F) 58 (M)*	Reaction to social challenge (pre and post measures)	No gender differences in FXS participants.
Hall, Lightbody & Reiss (2008)	Human	29 (F) 31 (M)	Collection at four time points during evaluation day	No main effect of gender
Wisbeck et al (2000)	Human	7 (F) 8(M)	Typical day Circadean rhythm (average 2 days)	No male and female difference.

			Experimental day circadian rhythm (involves novelty and social challenges)	Males significantly higher than females 30m post-stressor and before bedtime.
Markham et al (2006)	Mouse	8-12 per group	Response to acute stressor (restraint)	Different patterns of response and recovery to 30m of restraint stress. Males show protracted return to unstressed baseline; females show protracted rise. Peak secretion does not differ.

Table 5

Studies assessing associations between cortisol and behaviour in individuals with Fragile X Syndrome

Study	Behavioural measure			Association of behaviour with cortisol?			
	Topic	Method	Typical Day	Experimental Day			Other
				Pre-challenge	Reactivity	Post-challenge	
Hessl et al (2002)	Problem Behaviour	Child behaviour checklist (CBCL; Achenbach, 1991). Total and sub-scale scores. Controlled for other factors associated with behaviour problems (See full text)	*Female.* Typical day composite significantly positively correlated with attention problems.	-	-	-	*Males.* Composite cortisol level (unspecified) accounted for 8% of variance in total behaviour problems. Higher levels were associated with increased behaviour problems, especially withdrawn behaviour. *Female.* Cortisol levels account for 14% of variance in behaviour problems. Evaluation composite significantly positively correlated with

							social and attention problems.
Hessl et al (2006)	Social Escape	Measurement of gaze, vocal quality, discomfort and non-verbal task avoidance during social challenge.	-	-	Higher cortisol reactivity controlling for pre-challenge levels) associated with more gaze avoidance in siblings but opposite pattern in FXS (blunted response associated with increased gaze avoidance) for both males and females. No other associations found.	-	-
	Problem Behaviour	Aberrant behaviour checklist (ABC; Aman et al, 1985); CBCL and Autism Behaviour Checklist (Krug, Arick, & Almond, 1993)	-	-	Increased cortisol reactivity associated with increased sensory and social relation problems in FXS (no other associations). No associations in sibling group.	-	-

Hall et al (2006)	Social escape	Measurement of gaze, refusals, face-hiding, eye-rubbing, hand-biting, fidgeting, leaving chair during social challenge.	-	In males, increased cortisol associated with decreased eye contact and increased fidgeting. No association with other social escape behaviours or number of problem behaviours seen.	-	-	-
Hall et al (2008)	Autistic Behaviour	Autism Diagnostic Observation Schedule-General (ADOS-G; Lord, Rutter, DiLavore, & Risi, 2002).		In males only, more autistic behaviour associated with lower cortisol.	-	-	-
	Compulsions	Compulsive Behaviour Checklist (Bodfish, Crawford,Powell & Parker, , 1995)	-	No association cortisol and prevalence of compulsions.	-	-	-

	Self-Injurious Behaviour (SIB)	Self-injury checklist (SIB-C; Bodfish et al, 1995)	-	No association between cortisol and prevalence or number of forms of SIB.	-	-	-
Roberts et al (2009)	Autistic Behaviour (AB)	Scores on Childhood Autism Rating Scale (CARS; Schopler, Reichler, & Renner, 1986)	-	No associations	Decreased cortisol change associated with increased autistic behaviour in FXS+ASD (only)	No associations	-
	Social approach	Social Approach Scale- modified (Goldsmith & Lemery, 2000; Roberts et al, 2007): Initial and familiar approach (physical movement, facial expression & eye contact)	-	No associations in FXS group. In TD group increased cortisol associated with increased facial and eye contact during familiar social approach.	No association in FXS group. In TD group increased cortisol change associated with increased facial and eye initial social approach (no other associations)	FXS+ASD higher post-challenge cortisol associated with decreased initial physical approach.	-
Scherr et al (2016)	Verbal working memory	Score on Memory for Words Sub-test of *Woodcock–Johnson Tests of Cognitive Abilities, Third Edition* (WJ-III, Woodcock,	-	Higher baseline cortisol was associated with poorer performance on memory for words working	No significant association.	No significant association.	-

		McGrew, & Mather, 2001)		memory test, for both groups.			
	Verbal working memory	Auditory working memory sub-test of *Woodcock–Johnson Tests of Cognitive Abilities, Third Edition* (WJ-III, Woodcock, McGrew, & Mather, 2001)	-	Increased baseline cortisol associated with decreased performance in the FXS group, only.	No significant association.	Overall fixed effects for auditory working memory and cortisol change was significant, there were no significant effects of cortisol change or group	-