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Physiological regulation and social-emotional processing in female carriers of the *FMR1* premutation

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Abstract

The *FMR1* gene is associated with a wide range of clinical and cognitive phenotypes, ranging from intellectual disability and autism symptoms in fragile X syndrome (caused by the *FMR1* full mutation), to a more varied, and still poorly understood range of clinical and cognitive phenotypes among carriers of the gene in its premutation state. Because the *FMR1* premutation is relatively common among women (as high as 1 in 150), investigations of its phenotypic impact could have broad implications for understanding gene-behavior relationships underlying complex human traits, with potential clinical implications. This study investigated physiological regulation measured by pupillary responses, along with fixation patterns while viewing facial expressions among women who carry the *FMR1* premutation (PM group; n=47), to examine whether the *FMR1* gene may relate to physiological regulation, social-emotional functioning, and social language skills (where subclinical differences have been previously reported among PM carriers that resemble those documented in autism-related conditions). Relative to controls (n=25), the PM group demonstrated atypical pupillary responses and fixation patterns, controlling for IQ. In the PM group, pupillary response and fixation patterns were related to social cognition, social language abilities, and *FMR1*-related variation. Results indicate a pattern of atypical attention allocation among women who carry the *FMR1* PM that could reflect different emotion-processing strategies mediated by autonomic dysregulation and the *FMR1* gene. These findings lend insight into the *FMR1* gene's potential contributions to complex human traits such as social emotional processing, and social language.

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Keywords

Fragile X Syndrome; *FMR1* premutation; social cognition; physiological regulation; Autism Spectrum Disorder; Endophenotype

Fragile X Syndrome (FXS) is a monogenic disorder caused by a mutation in the fragile X mental retardation gene (*FMR1*) on the X chromosome, involving a Cytosine-Guanine-Guanine (CGG) repeat expansion in the promotor region of the *FMR1* gene [1]. The full CGG expansion mutation (> 200 repeats) leads to methylation of *FMR1*, shutting down production of the fragile X mental retardation protein (FMRP), leading to the cognitive-behavioral phenotype in FXS, including intellectual disability, social cognitive impairments, and ASD-related symptoms. The estimated prevalence of the full *FMR1* mutation is 1 in 4,000 males and 1 in 7,000-10,000 females [2,3]. The *FMR1* premutation is relatively common, occurring in approximately 1 in 150-250 women and 1 in 468 men and is identified by a CGG repeat length between 55-200 [4,5]. In its premutation state, expression of *FMR1* is dysregulated, including reduced FMRP and increased levels of mRNA, which have been hypothesized, along with the length the CGG expansion, to contribute to clinical-behavioral and cognitive features [6–8].

While the *FMR1* PM is not associated with the same clinical-behavioral impairments as FXS, a number of behavioral and neuropsychological phenotypes appear to be impacted by the *FMR1* PM, including differences in social-emotional functioning and social cognition more broadly [9–14]. Specifically, males who carry the *FMR1* PM demonstrate decreased social-cognitive abilities [10], difficulties with social information processing, and atypical social communication skills [15]. Further, elevated rates of subclinical ASD-related personality and language traits (e.g., social reticence, rigidity, social language differences) have also been reported in females with the *FMR1* PM [9,13]; together with elevated rates of comorbid ASD in FXS, findings suggest that *FMR1*-related variation may contribute to ASD-related phenotypes.

Considerable evidence exists that autonomic dysregulation, as characterized by hyperarousal, is evident in FXS, and is associated with social phenotypes (e.g., impaired social communication) [16–26]. Importantly, similar patterns of autonomic dysregulation have been documented in ASD, including atypical pupillary responses, heart rate, and vagal tone in social settings, which is thought to relate to broader social impairments [27,28,26,29–31]. Similarly, other single-gene disorders (e.g., Rett Syndrome, Angelman Syndrome) associated with ASD also display autonomic dysregulation which could be related to underlying genetic variations and relate to ASD symptomatology [32–34]. Results documenting autonomic dysregulation related to ASD symptomatology are consistent with the polyvagal theory, which suggests that autonomic regulation impacts social engagement through neuroanatomical connections between the vagus nerve and associated cranial nerves [35–40]. Atypicalities in these systems could provide an explanatory mechanism for understanding how autonomic regulation may impact ASD-related social phenotypes that span diagnostic boundaries.

Some evidence also exists to suggest that atypical autonomic regulation is present in the *FMRI* PM and may relate to subtle social-cognitive differences that have been documented in this group. Hessel et al. (2007) found that male carriers of the *FMRI* PM demonstrated dysregulated arousal responses, as measured by decreased skin conductance while greeting an unfamiliar examiner, along with decreased brain activity in areas of the brain implicated in social cognition while viewing fearful faces (i.e., amygdala, orbitofrontal cortex, superior temporal sulcus). Given our understanding of the aforementioned brain regions, it could be hypothesized that diminished neural responses to social stimuli might mediate downstream physiological responses and social cognition in the PM [41]. In a study examining heart rate and vagal tone during rest (both key measures of autonomic regulation), female carriers of the *FMRI* PM displayed dampened vagal tone, which has been associated with decreased emotional regulation, affective processing, and social language abilities [42–44,35,45,46]. A subsequent study also demonstrated that decreased vagal tone at rest in female carriers of the *FMRI* PM was associated with decreased social language abilities, but not with increased levels of anxiety [12]. Though carriers of the *FMRI* PM do not appear to display the hyperarousal evident in FXS, together, this evidence suggests that autonomic dysregulation may be associated with atypical social-cognitive and social language abilities linked with variations in the *FMRI* gene.

While autonomic regulation is typically measured through skin conductance or cardiac variables, pupillary dilation can also serve as a measure of sympathetic and parasympathetic nervous system activation [47,31]. Importantly, in addition to providing information on autonomic regulation, pupil dilation provides information on resources dedicated to attentional allocation and cognitive processing [48,49]. Specifically, pupil dilation is hypothesized to reflect activation of the locus coeruleus, which is a brain region intrinsically linked to attention and arousal networks [50,51]. Previous studies employing task-evoked pupillometry have also linked pupillary responses to social-emotional functioning, suggesting that measuring pupillary responses may be a reliable indicator of autonomic regulation and downstream behavior [52–54].

Further, studying changes in pupillary response over time (e.g., through growth curve analyses), may reveal important task-related attentional and autonomic arousal patterns over a continuous time-course [55,56]. For instance, applying different orthogonal polynomial terms captures the functional form (i.e., constriction and dilation) of the pupil response and can be used to understand nuanced changes in autonomic arousal. For example, Kuchinsky et al. (2014) utilized growth curve analyses to demonstrate change in pupil dilation during a sustained listening task, showing that cognitive load appeared to be reflected by time-sensitive changes in pupillary dilation (changes which would not have been detected using only traditional pupillary response variables such as mean pupil response, maximum pupil response, and latency to peak pupil response). These findings have been extended to comparable paradigms in the auditory domain, where findings have added considerably to understanding of arousal in response to sustained listening tasks with different signal-to-noise ratios [57–61]. Growth curve analysis can therefore serve as an effective tool for characterizing time-sensitive behavioral and biological phenomena, and may be particularly apt for characterizing differences in the pupillary response, though this analytic approach has yet to be applied to studies of pupillary response in *FMRI*-related conditions or ASD.

Evidence of atypical macro-level pupillary responses (e.g., mean and maximum response), linked with differences in social-emotional and communicative impairments in FXS implicates the *FMR1* gene in important pathways linking physiology and behavior. In this study we further explore this connection, by examining pupillary response in females who carry the *FMR1* PM while viewing emotional faces using the NimStim Facial Stimulus Set [62], studying both macro- and micro-level variables to characterize pupillary response across emotions of happy, fear, and calm. We also examined fixation patterns and duration on emotionally salient areas of interest (AOIs), and whether fixation patterns relate to pupillary response patterns. Finally, we explored social-cognitive and social language abilities as potential correlates of pupillary response and fixation patterns, for insights into links between physiological mechanisms and social cognition that may be tied to *FMR1* variation. Studying such links among PM carriers offers a window into the relationship between expression of the *FMR1* gene and FMRP and social-cognitive phenotypes. Therefore, we predicted that carriers of the *FMR1* PM would demonstrate atypicalities in both fixation patterns and pupillary response compared to controls, and that these atypicalities would relate to social cognition, social language abilities, and molecular-genetic expression of the *FMR1* gene.

Methods

Participants

Participants included 47 adult females with the *FMR1* PM (PM group) and 25 male and female controls without a family history of FXS, ASD, or related neurodevelopmental disabilities (see Table 1). Carrier status was confirmed through analyses of CGG repeats. The range of CGG repeats in the PM group was 59-125 repeats ($M = 91.03$, $SD = 18.87$). All protocols were approved by Northwestern University's Institutional Review Board.

Full scale IQ was determined using the Wechsler Abbreviated Scale of Intelligence (WASI) [63]. Participants were selected to participate in this protocol if they had a full scale IQ of at least 80 and were younger than 65 years old. The PM group displayed significantly lower full scale ($p = .02$) and performance IQ than controls ($p = .002$). Therefore, all analyses control for IQ when indicated. Groups did not significantly differ in chronological age ($p = .32$). Given known phenotypic and genetic differences between male and female carriers of the *FMR1* PM (where females carry an unaffected X chromosome), this study only included female carriers of the *FMR1* PM [64]. Both males and females were included in the control group, and because no sex differences were detected in demographic or experimental variables (i.e., $ps > .50$, age, IQ, pupillary response and fixation), males and females were combined in analyses. Prior studies examining pupil response to affective images have commonly combined males and females in analyses [47,65,66]. Participants were screened for use of psychotropic medications that may affect pupillary response and facial processing [67–69]. In the PM group, 32% of participants reported that they were prescribed antidepressant medication. T-tests revealed no significant differences between the PM group participants who reported taking medication and those who did not ($ps > .25$), although individuals taking antidepressant medications demonstrated a higher full scale IQ ($M = 111.87$, $SD = 11.40$ vs. $M = 108.18$, $SD = 9.51$, respectively; $p = .043$). Subsequent analyses

control for IQ, and therefore the PM participants prescribed antidepressant medication are combined with those who were not.

Eye Tracking Stimuli

Participants viewed a set of 60 faces (20 happy, 20 calm, and 20 fear) from the NimStim Facial Stimulus Set [70,62]. Trials were conducted as follows: 1) An inter-stimulus grey screen was presented for 0.5, 1.0, or 2.0 seconds, 2) a scrambled face image controlled for luminance was presented for 1.0 second, 3) the face image was presented for 3.0 seconds. The task was conducted in a dimly lit room, with light no greater than 10 lux whenever possible to allow for maximum opportunity for pupil dilation. Participants for which the room was greater than 10 lux (30% of the sample) did not display significantly different overall pupil responses than participants with lux lower than 10 ($ps > .23$). Eye tracking testing procedures mirror methodologies as outlined in previous studies of autonomic arousal in comparable populations [70,71].

Data Processing

Pupil data reduction—Pupil diameter and gaze fixation data were recorded, exported, and pre-processed on a Tobii T60 eye tracker through the Tobii system [72]. Data were sampled at 60Hz (every 16.67ms); all relevant eye tracking variables (e.g. pupil diameter, fixation location) were exported from Tobii at the same level of sampling. Raw data cleaning occurred using a customized script in SPSS 24 (unpublished). Following procedures in previous eye-tracking work, when only one eye was successfully sampled, linear regression was used to predict the missing pupil diameter, following established procedures [73,74]. Pupil diameter was then averaged from both eyes to create a mean for analysis. Consistent with previous eye tracking methodology, for brief intervals in which both eyes were missing pupil diameters, the average pupil diameter was linearly interpolated when gaps in the data were less than 350ms and the data tracked before and after the gap were stable [73]. Linear interpolation was only conducted when pupil diameter pre- and post-gap were available for at least 50% of the samples and the sample was twice as long as the total gap length. Extreme sample-to-sample pupil changes (e.g. change >2 SD) were eliminated from the dataset as they are likely to be due to partial blinks during the trial [75,30,74]. Trials were discarded if pupil diameter was not present for the last 100ms of baseline pupil diameter calculation and if less than 50% of the trial sample had valid pupil diameter measurements after the linear interpolation [76].

Macro-level pupil response variables—To characterize change in pupil diameter in response to the happy, calm, and fear conditions the following macro-level variables were derived: baseline pupil diameter, mean pupillary response, pupil diameter difference, maximum pupil response, and latency to maximum pupil response. All variables were averaged across conditions (i.e., happy, fear, calm) and across all trials. The derived macro-level pupil response variables are as follows:

- 1) *Baseline pupil diameter* was derived from the final 200ms of viewing the scrambled face image that precedes the face image. This variable was primarily used for data quality control.
- 2) *Mean pupillary response* was determined by averaging the pupil diameter for

each face trial and subtracting the previously calculated baseline diameter. Further, the pupil diameter difference from baseline was obtained for each 100ms of the face image presented, which was then used in the micro-level time series analyses. 3) *Maximum pupil response* was calculated based on the greatest positive change from the baseline pupil diameter for each face trial. 4) *Latency* to maximum pupil response (ms) was derived from the interval between initial presentation of the stimuli to the point of maximum pupil response relative to baseline.

Micro-level pupil response variables—To examine change in pupillary dilation over time, the change in pupil diameter from the baseline stimuli was calculated for every 100ms of the 3.0s trials. The average difference for each 100ms was then averaged for each emotion condition separately as well as altogether for each participant's valid trials.

Fixation data reduction—Using the Tobii I-VT filter set at a velocity threshold of 35° per second, available within the Tobii processing program, a fixation was determined as any consistent gaze for 100ms or greater. There was no set maximum fixation, and there was no fixation present that was greater than two standard deviations outside of the average fixation length. Fixations were merged if adjacent fixations were varied by less than 0.5° and were within the set 100ms fixation window. Using the x- and y-coordinates of each eye, fixation data was determined for each window. Following Wass, Smith, & Johnson (2013), if fixation data for a participant was missing for no longer than 150ms, the gap was linearly interpolated. However, if fixation x- and y-coordinates for one eye was missing, no interpolation occurred to determine fixation based on the coordinates of the other eye. Noise reduction occurred using a moving average window of three samples [77].

Fixation variables—AOIs were determined prior to data exportation and processing, and included regions of the eyes, nose, and mouth for each face stimulus. The fixation variables were averaged by emotion condition and across all emotion conditions. Two key variables were derived to analyze fixations: time to first fixation for each AOI and proportion fixation duration on each AOI. The fixation variables are as follows: 1.) *Time to first fixation* was determined by measuring the time elapsed from presentation of the face stimulus until the first fixation of at least 100ms on an AOI. 2) *Proportion of fixation duration* on each AOI was derived by summing the fixation duration of each AOI and dividing it by the total duration of all fixations on each trial.

Data quality—Trials were considered invalid if fixation data were missing for over 50% of each trial and if more than 50% of their trials were invalid [78]. To verify comparability of data quality between groups, independent samples t-tests were conducted on relevant data quality variables: pupil data loss, fixation data loss, number of valid trials, baseline pupil average (as determined by the last 200ms of the scrambled face), and overall fixation duration, revealing highly similar data quality between groups. Overall pupil data loss was not significantly different for the PM group ($M = .04$, $SD = .09$) and control group ($M = .03$, $SD = .04$; $t(65) = .043$, $p = .67$), and fixation data loss was similar between groups (PM group: $M = .09$, $SD = .08$; control group: $M = .10$, $SD = .07$; $t(55) = -.37$, $p = .71$). The number of valid trials did not differ between groups (control group: $M = 47$, $SD = 19.45$;

PM group: $M = 51$, $SD = 14.67$) ($t(61) = -0.93$, $p = .36$). The baseline pupil diameter prior to presentation of the stimuli was no different between the PM ($M = 2.87$, $SD = .34$) and control groups ($M = 2.84$, $SD = .33$) ($t(52) = .45$, $p = .66$). Finally, overall fixation duration (ms) did not significantly differ between the PM ($M = 2200$, $SD = 361$) and control groups ($M = 2087$, $SD = 381$) ($t(47) = 1.23$, $p = .23$). Due to data loss eight PM group participants and three control group participants were excluded. On average participants in both groups contributed 53 valid slides (out of 60). Correlational analyses utilized all participants with valid data to maximize the sample for examining clinical-behavioral correlates.

Social Cognition, Social Language, and Molecular Genetic Correlates

Social cognition—Social cognition was assessed using the Reading the Mind in the Eyes Task [79], an advanced theory of mind task that requires determining complex thoughts and feelings based on photos of the eye region of the face. A subset of participants also completed an Emotion Identification task that taps reliance on facial expression to discern the emotional content of a scene. In this task, movie scenes are shown with and without faces depicted, and participants are asked to label the emotion best conveyed by the scene in each condition [80]. Analyses examine the difference in accuracy recognizing the emotional content of the scene in images with and without the faces present. A larger score indicates greater reliance on facial expressions when discerning emotional content, as it demonstrates improvement in the ability to identify emotional content when facial expressions are present.

Social language—Social language ability was assessed using the Pragmatic Rating Scale (PRS) [81]. The PRS was coded from a semi-structured conversational interview during which participants discussed their life history, relationships and interests. The PRS is comprised of 25 items which assess pragmatic language violations (e.g., tangential conversations, overly succinct responses, dominating the conversation, etc.). Each video was coded by two independent raters blind to group status, and consensus coded through discussion.

FMR1 Molecular Characterization

Blood samples were processed to determine CGG repeat length, quantitative FMRP (pg/ug), and activation ratio (i.e., the percentage of cells for which the active X chromosome contains an unaffected *FMR1* gene) (samples not available for 15 participants, for whom PM status was confirmed through review of medical records). FMRP was derived using the Luminex Technology immunoassay [82]. Primary lymphocytes were isolated from blood and stored at -80 degrees. For assay lymphocytes were thawed, spun at $4000 \times g$ for 10 minutes, washed, lysed in the presence of protease inhibitors, rotated overnight and spun at $16,000 \times g$ for 20 minutes. Quantification of total protein was done with a bicinchoninic acid protein assay kit, in duplicate for each lymphocyte sample. FMRP was quantified by using a Singleplex Luminex protein immunoquantification assay. The FMRP bound to x-map beads was tagged by two antibodies, the first is rabbit anti-FMRP R477 and the second is a phycoerythrin-conjugated goat anti-rabbit IgG, which generates a signal detected in the Luminex FlexMap 3D machine. Calculation of FMRP is conducted compared to a standard reference sample of recombinant fusion protein carrying short domains of FMRP and GST-

SR7. The activation ratio was determined by Southern blot as described by Berry-Kravis, Potanos, Weinberg, Zhou, & Goetz [83].

Data Analysis Plan

Primary analyses examined group differences in macro- and micro-level pupil response variables, along with fixation patterns while controlling for IQ. All results presented are those which hold when controlling for IQ. Exploratory correlations were also conducted to understand potential associations between pupillary responses, fixation patterns, social cognition language, and *fMRI*-related variation within the PM group. Based on findings from Farzin et al. (2009) showing that differences in pupillary responses in children with FXS were driven by happy and fearful emotional expressions, data were analyzed both within each specific emotion and also averaged across emotions.

To control for type 1 error, Tukey corrected post-hoc tests were conducted for analyses of group differences across pupil response and fixation data. Partial eta-squared were reported along with significance values to demonstrate effect size. Because correlational analyses were exploratory and based on a reduced sample size, no corrections were made for multiple correlations. Exploratory correlations were considered marginal if they had a p-value between .05-.10. Therefore, the medium ($r = .30$) to large ($r = +.50$) effect sizes of the correlation coefficients can be interpreted in addition to the raw p-value [84].

Pupillary response—Macro-level data analysis assessing differences in mean pupil response, maximum pupil response, and latency was conducted using separate between-subject ANOVAs, controlling for full scale IQ, by condition and across all conditions.

Micro-level pupil response over the 3.0s timecourse of stimulus presentation was analyzed using growth curve analysis, consistent with prior work [57,58]. This technique is more powerful than time-binned ANOVA comparisons as it captures the continuous nature of time series data [55,56]. Group differences for change in pupil diameter were examined for the three specific emotions, and across all emotions averaged using a fourth order (quartic) orthogonal polynomial regression equation, co-varying for IQ, using adapted code in *R* Studio [55]. A quartic level equation was applied given its established utility for modeling the functional form of pupillary dilation and constriction over the presentation of the stimuli [57,58,85,60,61]. In a quartic level model, Kuchinsky et al. (2013, 2014) have described the linear term as the overall change in pupil dilation, the quadratic term as reflecting the transience of the response, the cubic term as reflecting the timing, and the quartic term as the presence of tail end fluctuations. Importantly, though studies differ in their inclusion of higher-order polynomial terms, a quartic model was chosen given our theoretical interest in the information reflected by each polynomial term (i.e., the timing and robustness of the initial pupillary dilation, subsequent pupillary constriction, and the presence of tail-end fluctuations as represented by the quartic level term).

Fixation patterns—The proportion fixation duration and time to first fixation across AOIs were analyzed to compare differences in visual attention between groups. Separate between-subjects ANOVAs, controlling for IQ, were the primary method of analyzing differences in

macro-level proportion fixation duration and time to first fixation. These analyses were conducted for each AOI and were averaged by condition and across all conditions.

Links between pupil response and fixations—Associations between the macro-level fixation variables and the pupillary response variables were investigated using Pearson correlations within groups. Of note, microanalyses of timecourse pupillary responses were not included in subsequent correlational analyses given that the polynomial transformations were of time rather than change in pupil response. Because IQ was not correlated with any pupillary or fixation variable it was not included as a covariate.

Associations between fixation and pupillary response and clinical-behavioral features and FMR1 variation

Pearson correlations were used to examine associations between pupillary macro-level variables and the Reading the Mind in the Eyes Task (Control Group $n = 24$; PM Group $n = 43$) due to the fact that the data was normally distributed and continuous (i.e., percent correct). Spearman correlations were used for the secondary Emotion Identification task within scenes (Control Group $n = 12$; PM Group $n = 25$) were conducted in the PM group and control group separately. Spearman correlations were used rather than Pearson because the value provided in the secondary Emotion Identification task is ordinal. IQ was included as a covariate for correlations with the Reading the Mind in the Eyes task, given its positive association with performance in the PM group ($r = .38, p = .13$). Pearson correlations covaried for IQ were also conducted for associations with social language abilities as measured by the pragmatic rating scale. Social cognitive and social language data were available on a smaller subgroup of controls (number of participants differ by task), who were not administered the full battery of project tasks due to time constraints in participation. Correlations within the control group are still included in this manuscript to demonstrate the contrast between the PM group, however, the focus of the results is on the PM group. To investigate *fMRI*-related variation, a series of regressions were conducted within the PM group between quantitative FMRP, number of CGG repeats controlling for activation ratio, and overall pupil response and fixation variables within all emotions averaged together. Further, the relationships between CGG repeats and eye-tracking variables were also explored accounting for activation ratio by including an interaction term within the regression models [86,87].

Results

Macro-Level Pupil Response

Averaged over the entire three second trial for all emotion conditions, there was no significant group difference for mean pupillary response ($F(1, 190) = .92, p = .34, \eta^2 = .000$) or latency to peak pupillary response ($F(1, 190) = 0.09, p = .77, \eta^2 = .001$). However, the PM group showed a smaller peak pupillary response than the control group ($F(1, 190) = 4.02, p = .046, \eta^2 = .023$) (See Figure 1). There were no main effects of emotion condition for mean pupil response, maximum pupil response, or latency to peak pupil response ($ps > .14$).

Micro-Level Pupil Response

A significant group difference was detected for the cubic polynomial term, indicating atypical timing of the pupillary response in the PM group, relative to controls in all of the emotion conditions averaged together ($Estimate = -.67, p = .002$) as well as in the calm and fear faces (calm: $Estimate = -.50, p = .012$; fear: $Estimate = -.40, p = .035$). Averaged together, there were no other significant differences present in any other polynomial term ($ps > .10$; see Table 2). In response to the calm face, the PM group demonstrated a smaller pupil diameter (intercept; $Estimate = .51, p = .002$), and marginally less change overall (linear polynomial; $Estimate = .70, p = .093$). The PM group also showed a more transient pupil response (quadratic polynomial; $Estimate = -.56, p = .021$) than the control group. No other polynomial terms were significant amongst the other emotional faces. Growth curve model parameters, and patterns of pupillary response by polynomial terms are presented in Table 2 and Figure 2, respectively.

Fixation Patterns

Proportion fixation duration—There was no significant main effect of group for overall proportion of fixation duration ($F(1, 195) = .37, p = .54, \eta^2 = .001$), but there was a significant main effect for AOI type ($F(2, 195) = 231.25, p < .001, \eta^2 = .703$), and a significant two-way interaction between group and AOI type ($F(2, 195) = 151.43, p < .001, \eta^2 = .608$). Post-hoc within-group analyses revealed that whereas controls fixated more on the eyes compared to the mouth, the mouth compared to the nose, and the eyes compared to the nose ($ps < .001$), the PM group demonstrated a different pattern, with more fixations on the mouth compared to the eyes and nose, and more on the eyes than the nose ($ps < .001$). The PM group fixated less on the eyes and nose and more on the mouth compared to controls ($ps < .01$; see Figure 3).

Time to first fixation—Time to first fixation differed by group ($F(1, 194) = 25.24, p < .001, \eta^2 = .115$) and AOI ($F(2, 194) = 10.28, p < .001, \eta^2 = .095$). A two-way AOI by group interaction also emerged ($F(2, 194) = 22.83, p < .001, \eta^2 = .190$). Post-hoc analyses revealed that the PM group fixated more slowly on the nose in the calm condition ($p = .044$), and more quickly to the mouth in all emotion conditions ($ps < .001$).

Correlations between Pupil Response and Fixations

Sporadic correlations were detected between macro-level pupil response and fixation variables. There were no significant correlations in the PM group or control group averaged across emotions for the eyes and mouth for proportion fixation duration. In the happy face, the PM group and control group demonstrated inverse associations between proportion of fixation duration on the mouth and eyes and pupil diameter, such that a larger pupil diameter in the PM group was associated with increased time spent fixating on the mouth ($r = .27, r^2 = .073, p = .094$) and less time spent fixating on the eyes ($r = -.33, r^2 = .109, p = .034$), whereas a larger pupil diameter in controls was associated with increased time spent fixating on the eyes ($r = .48, r^2 = .230, p = .025$), and less time spent fixating on the mouth ($r = -.45, r^2 = .203, p = .036$). For time to first fixation, in both the PM and control group a smaller pupil response was associated with a longer time until their first fixation to the eyes,

specifically in the calm face ($rs < -.33$, $r^2s > .109$, $p < .10$). There were no other significant correlations in the PM group or control group across emotions for the eyes and mouth for time to first fixation.

Social-Cognitive and Language Correlates of Pupillary Response and Fixation Patterns

Social cognition—In the PM group, better performance on the Reading the Mind in the Eyes task of social cognition was associated with longer time to fixate on the eyes ($r = .32$, $r^2 = .10$, $p = .040$) and quicker fixation to the mouth (though marginal, medium effect size; $r = -.27$, $r^2 = .073$, $p = .080$), controlling for IQ and across all emotions. The control group also demonstrated an association between better Reading the Mind in the Eyes performance and longer time to first fixation on the eyes ($r = .43$, $r^2 = .185$, $p = .038$) across emotions.

In the PM group, on the Emotion Recognition task, stronger reliance on facial information to identify neutral emotions was associated with less fixation time on the eyes ($r = -.42$, $r^2 = .176$, $p = .036$), whereas a stronger reliance on facial information to identify surprised faces was associated with more fixation time on the mouth ($r = .46$, $r^2 = .212$, $p = .020$) and marginally quicker first fixations towards the mouth ($r = -.36$, $r^2 = .130$, $p = .078$). The control group demonstrated a similar relationship—stronger reliance on facial information to identify happy and afraid faces was associated with less time spent fixating on the eyes ($rs < -.60$, $r^2s > .360$, $ps < .50$) and more time spent fixating on the mouth (happy only; $r = .73$, $r^2 = .533$, $p = .007$). No other significant correlations were detected.

Social language—In the PM group, increased latency until maximum pupil response and an increased pupil response was also marginally associated with increased social language difficulties ($rs > .30$, $ps < .10$). Further, in the PM group, there was a trending association with a medium effect size between quicker fixations to the eyes in the calm face ($r = -.269$, $r^2 = .072$, $p = .103$) and longer time to first fixation on the mouth ($r = .330$, $r^2 = .109$, $p = .038$) and greater social language difficulties. The control group also demonstrated a trending association between longer time until first fixation on the mouth and greater social language difficulties ($r = .386$, $r^2 = .149$, $p = .084$).

FMR1-related variation—Patterns of associations were inconsistent for each emotion condition, so associations with pupil responses and fixation patterns overall are presented by emotion when significant. Overall, fewer CGG repeats with activation ratio was associated with shorter time until first fixation on the mouth ($r^2 = .30$, $p = .08$). While viewing the calm face, longer time to first fixation on the eyes was marginally associated with greater CGG repeats ($r^2 = .084$, $p = .10$). While viewing the happy face, a greater average and maximum pupil response was associated with greater FMRP ($r^2s > .10$, $ps < .10$) and fewer CGG repeats with activation ratio ($r^2 = .30$, $p = .07$). Overall correlations between CGG repeats and quantitative FMRP are presented in Figure 4.

Discussion

This study investigated macro- and micro-level pupil dilation characteristics, together with fixation patterns while viewing emotional facial expressions in carriers of the *FMR1* premutation relative to controls, controlling for IQ. We also explored potential links with

social cognition and social language, as well as *fMRI*-related variation. Findings revealed autonomic dysregulation and differences in attentional allocation (i.e., fixation patterns showing more focus on the mouth and less on eyes and nose than controls) across different emotional facial expressions in the PM group. Interestingly, this atypical fixation pattern in the PM group was associated with *better* performance on social-cognitive tasks and stronger social language abilities. Shorter time to first fixation on the mouth and longer time to first fixation on the eyes as well as increased pupillary response were related to fewer CGG repeats and increased FMRP. Together results suggest potentially important, and somewhat unexpected, profiles of autonomic regulation linked with atypical attentional allocation and social abilities in females carrying the *FMR1* PM.

Differences in autonomic regulation indexed through pupillary response among the *FMR1* PM add to a robust literature documenting hyperarousal and autonomic dysregulation in response to social stimuli associated FXS, which is similar to what has been documented in ASD. These findings also importantly extend prior reports of autonomic arousal differences in FXS, the PM, and ASD that have documented atypical responses to social stimuli through measurement of heart rate, vagal tone, and skin conductance [16,17,70,19,88,15,12,42,21,23,28,20,26]. The PM group in this study demonstrated a unique pattern of physiological arousal compared to what has been documented in ASD and FXS, where hyperarousal in response to social stimuli has typically been observed [28,70]. Further, in contrast to prior reports of dampened parasympathetic tone in females with the PM [12,42] and sympathetic tone in males with the PM [88], findings of a smaller maximum pupil response and a different trajectory of pupillary response over time suggest neither decreased sympathetic activation nor dampened parasympathetic activation, but rather, a possible imbalance.

Of note, the difference between the PM group and control group largely dissipated in the second half of the image presentation, which is consistent with previous research demonstrating robust pupillary responses occurring at around two seconds after image presentation [47]. The observation that the PM group demonstrated most striking differences at the onset of the image presentation suggests that the first seconds after image presentation may be important for understanding the differences in autonomic reactivity associated with the *FMR1* premutation. Specifically, in spite of similar initial pupil dilation in the PM and control groups (suggesting adequate sympathetic and parasympathetic activity), the increased constriction observed immediately following initial dilation in the PM group may reflect increased parasympathetic activity and decreased sympathetic activity. Greater parasympathetic activation (i.e., increased pupillary constriction) is typically linked with more favorable outcomes related to mood, behavior, and prosocial interactions [35,40,89–91], although some studies suggest a less straightforward relationship [92–94]. For instance, Kogan et al. (2014) found that typically developing participants with a parasympathetic response closer to the mean, demonstrated increased prosocial relationships compared to participants with both increased and decreased parasympathetic activity. Eisenberg et al. (1995) found a different pattern in typically developing young girls, where those with higher parasympathetic activation demonstrated diminished social competence and increased behavioral regulation difficulties. These findings suggest that both increased and decreased parasympathetic activity may negatively impact social behavior and emotional regulation.

Interestingly, the differences in pupil response in the PM group were driven by the calm face rather than the more emotionally expressive happy and fear faces. The atypical sympathetic activation observed in response to the calm face is consistent with evidence that calm/neutral facial expressions may be more challenging to discern (versus basic emotions such as happiness and fear that are associated with easily recognizable and highly regular expressions) and thus require greater attentional resources [95,96]. Differences in fixation patterns in the PM group also suggest atypical processing of affective facial expression, and are in line with prior reports of gaze differences among PM carriers (Klusek et al., 2017) and in FXS (Farzin et al., 2009). Whereas a robust bias toward the eyes region is typically observed [97–99], the PM group fixated less on the eyes and more on the mouth as well as quicker to the mouth and slower to the eyes. This pattern resembles findings documented previously in ASD and among parents of individuals with ASD. In a task that tapped reliance on different regions of the face to discern emotional expressions, both individuals with ASD and parents showed a marked reduction in reliance on the eye region and increased utilization of the mouth region relative to controls [100–102]. Such similarities in gaze during social-emotional processing across conditions adds to existing work showing considerable phenotypic overlap in *FMR1*-related conditions and ASD [103,104,9,105], that could implicate *FMR1* in key ASD-related phenotypes.

Analyses of associations between fixation patterns autonomic arousal responses revealed further group differences. In the PM group, increased pupil response was associated with looking less at the eyes and more at the mouth, whereas inverse associations were found in the control group (i.e., greater pupil response observed with greater attention to the eyes). These patterns might suggest greater attentional resources recruited for processing information from the mouth region in the PM group, and eyes in the control group. In the *FMR1* PM, these interrelationships could reflect a dysregulated arousal network underlying different social perceptual strategies to enhance social cognition and social language abilities, and are intriguing in light of findings that atypical fixation patterns were associated with better social cognitive performance and stronger social language skills, perhaps suggesting a compensatory pattern of fixation and attention when processing affective facial expressions. It may be that individuals with the PM find social-emotional information from the eye region less informative during social-emotional interactions, and glean more meaningful information from the mouth region.

Finally, findings that increased CGG repeats and decreased quantitative FMRP levels were associated with decreased pupil response and increased time until first the first fixation on the mouth region suggest that *FMR1*-related variation in the PM may be importantly linked to autonomic arousal and fixations to social stimuli, consistent with previous research demonstrating links between *FMR1* expression and processing of social information [16,18,21].

Limitations, Strengths, and Future Directions

Potential limitations that should be considered in interpretation of results include the inclusion of females only, and the study's relatively modest sample size. Although this study included female PM carriers exclusively to avoid the confound of sex (i.e., the presence of

an additional unaffected X chromosome and functional copy of *FMRI* among females), the inclusion of females only nonetheless limits generalizability of findings. And, whereas our sample size was comparable to those included in prior work (owing largely to the difficulty in ascertaining PM carriers, most of whom must be ascertained through a child with FXS, a rare condition), it will be important to replicate these findings with larger samples. It will also be informative to include multiple indices of physiological regulation within the same sample, to better understand potentially complex patterns of dysregulation indicated by different physiological metrics. For instance, incorporating additional analyses of the neuroendocrine system (i.e., cortisol) would provide another avenue for understanding how physiological interactions may modulate the interrelationships between arousal and social-emotional functioning.

An important contribution of this study is the further delineation of the role of the *FMRI* gene in physiological processes and its potential impact on downstream social cognition and subclinical social language differences that have been associated with ASD, highlighting the potential for the *FMRI* gene to affect these processes across diagnostic boundaries. Dysregulation in the autonomic nervous system has been called upon as an explanatory mechanism for the social impairments observed in ASD given repeated findings of associations between atypical physiological responses and social functioning [28,106,26,107]. Findings reported here that PM carriers showed atypical pupillary responses and fixation patterns in a social-emotional processing task provide evidence of associations between the *FMRI* gene and pupillary responses, which may be linked to downstream social abilities impacted in ASD, and suggest how the *FMRI* gene might be studied as a foothold to understand complex genetic influences on ASD-related phenotypes. Moreover, given similar patterns of autonomic dysregulation observed in ASD and FXS, future research examining potential overlapping physiological responses in parents of individuals with ASD and carriers of the *FMRI* PM may shed light on how autonomic dysregulation may contribute to ASD-related traits across diagnostic boundaries.

The application of growth curve analyses to examine continuous changes in pupil diameter over the trial duration built on prior work by providing a sensitive method for analyzing continuous data that is sensitive to small variations in responses over time [57,58,55,56], revealing important group differences not evident in macro-level variables. It could therefore be useful to apply similar methodologies and analyses to the study of physiological regulation and related biological phenomena supporting social-emotional functioning in *FMRI*-related conditions, to move beyond more global response averages that might obscure important patterns related to *FMRI* variation and clinical-behavioral phenotypic expression. An additional contribution of this study is the inclusion of social-cognitive and social language correlates, which permitted examination of connections between physiological and cognitive-behavioral phenotypes that are important for understanding the impact of *FMRI* mutations in carriers, and with potential for implications for ASD-related phenotypes as well.

In sum, this study builds upon the growing body of research highlighting autonomic dysregulation related to *FMRI* gene, in both its full and premutation states. Findings suggest that autonomic dysregulation may have an impact on important social phenotypes, even in a

population that does not exhibit clinical impairment. Overall, this study provides support for complex interrelationships between the *FMR1* gene, autonomic arousal, and social-emotional functioning in female carriers of the *FMR1* PM and presents several new directions for continued research aimed at connecting gene, physiology, and complex behavior that can help to explain both clinical impairment in ASD and variation in the general population alike.

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Figure 1. Macro-level pupil response variables: Mean pupil response, maximum (max) pupil response and time to maximum pupil response (latency) averaged across emotion conditions. Error bars represent one standard error (SE). Note: * $p < .05$.

Figure 2.

Micro-level analyses of pupil response over the 3.0 second timecourse for the happy, calm, and fear conditions as modeled by a 4th order polynomial regression equation. Error bars represent one standard error (SE).

Figure 3. Mean of proportion fixation duration for the mouth, eyes, and nose, for each emotion condition. Error bars represent one standard error (SE).

Figure 4. Correlations between CGG repeats, quantitative FMRP, fixation variables, and pupillary response variables. The colors are representative of the associated correlation coefficients.

Table 1.

Participant Characteristics.

Mean (*M*) and standard deviation (*SD*) for chronological age and IQ in the PM group and control group.

Group (n)	Chronological Age <i>M</i> (SD)	Full Scale IQ <i>M</i> (SD)	Performance IQ <i>M</i> (SD)	Verbal IQ <i>M</i> (SD)
Control (n = 25) M:F 10:15	41.72 (7.81)	116.48 (11.48)	117.82 (10.13)	111.62 (12.57)
PM (n = 47) all F	43.95 (10.71)	110.22 (8.88)*	109.38 (10.20)*	108.14 (11.32)

Note.

*
 $p < .05$, indicates difference from the control group

Table 2.

Growth Curve Analyses model parameters.

Model parameters, including the estimate, t-value, and p-value for the growth curve analyses.

	All			Happy			Calm			Fear		
	Estimate	<i>t</i>	<i>p</i>	Estimate	<i>t</i>	<i>p</i>	Estimate	<i>t</i>	<i>p</i>	Estimate	<i>t</i>	<i>p</i>
Control > PM												
Intercept	-.05	-.26	.80	.09	.55	.58	.51	3.09	.00**	-.11	-.54	.59
Linear	-.29	-.67	.50	.42	.64	.52	.70	1.68	.09 [†]	-.35	-.72	.47
Quadratic	.25	.70	.48	-.09	-.34	.73	-.56	-2.32	.02*	-.39	-1.17	.24
Cubic	-.67	-3.05	.00**	.07	.21	.84	.49	2.50	.01*	.40	2.11	.03*
Quartic	-.26	-.88	.38	-.09	-.48	.63	-.06	-.41	.68	.04	.78	.78

Note:[†]*p* < .10;**p* < .05;***p* < .01