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PRINCIPAL INVESTIGATOR: Gang Yao, Ph.D.

CONTRACTING ORGANIZATION: University of Missouri Columbia, MO 65211

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**Title:** Atypical Pupillary Light Reflex in Individuals With Autism  
**Authors:** G. Yao, S.E. Christ, J.H. Miles, D.Q. Beversdorf  
**Institution:** University of Missouri  
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### Abstract

Pupillary light reflex (PLR) refers to the involuntary response whereby the pupil size changes in response to a short flash light. In this project, we will evaluate the atypical dynamic pupillary light reflex (PLR) observed in children autism. In addition, we will develop an integrated PLR-fMRI protocol for imaging PLR associated brain activities. At the end of the 3rd project year, we have tested pupillary light reflex (PLR) and heart rate variability (HRV) in 304 participants including 152 children with ASD (the “ASD” group), 116 children of typical development (the “TD” group), and 36 children with other development disorders (the “NDD” group). The results showed that the ASD group had significantly longer PLR latency, reduced relative constriction amplitude, and lesser constriction/redilation time than those of the TD group. Similar atypical PLR parameters were observed in the NDD group. A significant age effect on PLR latency was observed in children younger than 9 years in the TD group, but not in the ASD and NDD groups. Atypical HRV parameters were observed in the ASD and NDD groups. A significant negative correlation existed between the PLR constriction amplitude and average heart rate in children with an ASD, but not in children with typical development. We also developed an integrated fMRI/PLR protocol and have obtained fMRI data for 33 adolescents with ASD and 27 typically developing adolescents without ASD.
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INTRODUCTION
In this project, we propose to further evaluate the atypical dynamic pupillary light reflex (PLR) observed in children autism. PLR refers to the involuntary response whereby the pupil size changes in response to a short flash light. There are two specific tasks in this 3-year project. In Task #1, we propose to test 200 human subjects including 100 children with autism (“ASD” group), 65 typically developing children (“TC” group), 35 children with early brain dysfunction unrelated to autism (“MR” group). In addition to the PLR test, each participant will be assigned a score as the likelihood to have ANS dysfunctions based on an extensive medical evaluation. The heart rate variability (HRV) data will also be obtained as a reference. In Task#2, we will develop a new integrated PLR/fMRI test and study PLR correlated fMRI in a total of 50 human subjects (25 children with autism and 25 typically developing children). We will test the hypothesis that the observed atypical PLR latency in individuals with autism is associated with abnormal cerebellum functions.

BODY:
For Task #1, we have tested pupillary light reflex (PLR) and heart rate variability (HRV) in 304 participants including 152 children with autism, 116 children of typical development, and 36 children with other development disorders. As described below, the current results confirmed atypical PLR and revealed significant different HRV in children with autism. In addition, we found significant correlation between PLR constriction and sensory behavior in the ASD group but not in typically developing children.

For Task #2, we successfully developed an integrated PLR and fMRI protocol and validated this new methodology in small sample adult participants. We then enrolled 33 participants with ASD and a demographically-matched comparison group of 27 participants without ASD. We also developed and validated a novel method for estimating the PLR curve and parameters based on data from the current eye tracking device. Our initial analysis using standard fMRI data processing and analysis techniques failed to reveal significant differences in PLR-related brain activity between the ASD and non-ASD groups. Presently we are re-analyzing the fMRI data using a novel, time-intensive approach which will hopefully allow us to better detect more subtle differences in brain activity.

Description of Task #1:
The PLR test protocol has been described in details elsewhere (Daluwatte et al., 2012). We measured the pupillary light reflex (PLR) under both dark-adapted and light-adapted conditions in each participant. The recorded pupil images were automatically processed to extract the pupilogram (change in pupil size with time shown in Fig.1). The following PLR parameters were measured: the initial pupil diameter D0, the maximal constriction diameter Dm, the PLR latency tL (interval between stimulus onset and beginning of constriction), the constriction time tc (interval between constriction

Fig.1. An illustration of the pupilogram and extracted PLR parameters. Please see text for explanations of each parameter.
onset and the maximal constriction), and the recovery time $t_r$ (interval between the maximal constriction and the recovery to half of the maximal constriction). The relative constriction (in percentage) was calculated as $(D_0^2-D_m^2)/D_0^2$.

We also monitored the participant’s heart rate during the entire test. A remote heart rate monitoring device (Polar RS800CXTM, Polar Electro Oy, Finland) was chosen to record QRS interval in real time. A chest strap with sensor and wireless transmitter attached is wrapped around the participant’s chest. The heart beat QRS signals transmitted from the chest strap is received and recorded by a watch-like device. The HRV dataset includes five standard HRV measures: standard deviation of normal-to-normal RR intervals (SDNN), root mean square successive differences (RMSSD), low-frequency component LF (0.04-0.15Hz), high frequency component HF (0.15-0.4Hz), and the ratio of LF/HF. The values are reported during five different time segments in the PLR test: before PLR test, during light-adapted PLR test, during dark-adaptation, during dark-adapted PLR test, and after all PLR test.

In addition to PLR and HRV, we collected a comprehensive questionnaire on each participant’s medical history including autonomic nervous system (ANS) functions, fever history, sleep disorders, sensory profile and medication.

**Results**
The major results have been recently published in Journal of Autism and Developmental Disorders (Daluwattee et al. 2013, attached). In brief, children with ASD exhibited significantly longer PLR latencies and lesser relative constriction amplitude than children of typical development (Fig. 2). The ASD group also had shorter constriction time and a shorter redilation time than those of the typically developing children (TC group). In addition, the PLR latency decreased from 6 to 8 years and reached a plateau thereafter in the TC group. This age effect did not exist in the ASD group at any stimulation conditions (Fig. 2c).

**Fig. 2.** Longer PLR latency (a) and lesser PLR constriction (b) in children with ASD than children of typical development obtained at various stimulation intensities. (c) PLR latency vs. age measured in the TC and ASD groups at light-adapted condition with stimulus intensity of LA872.1 cd/m². The lines are fitting results using an exponential decay function $y=a*\exp(-b*x)+c$. The error bars indicate the standard error.

Our investigation indicated that the group difference between ASD and TC is not caused by the different IQ distribution or by medications taken by the children with ASD. PLR constriction amplitude is an indicator of parasympathetic modulation (Clarke 2007). A lesser PLR constriction observed in children with ASD suggests lower parasympathetic modulation. The ASD group also showed a greater average heart rate than that of the typical controls (Daluwattee et al., 2013). Interestingly, a statistically significant negative correlation existed
between PLR constriction and average heart rate in the ASD group but not in the typically developing children. These findings suggest that an abnormality in the autonomic nervous system (ANS) is associated with ASD.

The age effect on PLR latency that we observe in typically developing children appears to reflect a normal neurodevelopmental progression. It is different from the visual system maturation characterized by pattern visual evoked potential (VEP), which stabilizes after 6 months of life (McCulloch and Skarf 1991). It appears similar to the white matter maturation trend revealed in diffuse-tensor MRI studies (Bashat et al. 2007). In those studies it appears that children with an ASD have accelerated white matter maturation before 4 years of age (Bashat et al. 2007; Weinstein et al. 2011), but this trend is reversed after 4 years of age (Vissers et al. 2012). Such change in trend is also similar to our observation in PLR latency (Fig. 2c). Future studies will be needed to determine exactly what neurodevelopmental process the PLR is documenting. Nevertheless, the absence of age-dependent change in PLR latency indicates that some normal neurodevelopmental trajectory is altered in children with ASD.

It is known that ANS is also involved in modulating sensory processing and sensory dysfunction has been widely reported in children with ASD. However, the potential association between physical measurements (e.g. PLR) and behavioral observations (e.g. sensory) has not been examined extensively in literature. Therefore we investigated the potential correlation between PLR and sensory measures in children with ASD. We found a weak but significant correlation between PLR constriction amplitude and sensory total score in the ASD group but not in typically developing children (Fig. 3). Lower PLR constriction amplitude suggests lower parasympathetic modulation. This observation implies that abnormal sensory responses in children with ASD could be associated with decreased parasympathetic modulation.

![Graph](image)

**Fig. 3.** The correlation between PLR constriction amplitude (at LA 8721.1 cd/m² stimulus intensity) and total sensory score in the (a) ASD and (b) TD groups. The Spearman rank correlation $r = 0.26$, $p < 0.01$ in the ASD group; $r = 0.003$, $p > 0.05$ in the TD group at LA 8721.1 cd/m². Lower sensory scores indicate greater atypical sensory behavior.

Our data analysis (Daluwatte et al. 2013) showed that frequency-domain HRV parameters appeared to change significantly when transiting between the rest and test phases in both the ASD and TD groups. Specifically, the HFN decreased during transition from a resting phase to a PLR test phase and increased during transition from a testing phase to a resting phase. The LF/HF showed a reversed trend. This observation is similar to the previously reported posture-induced HRV changes associated with orthostatic stress (Mukai and Hayano 1995). The PLR test requires the participant to incline slightly forward (~15°), and this posture
change can cause elevation in sympathetic tone due to muscle stress. Therefore a relevant question arises regarding whether stress can change PLR parameters. To address this, we investigated the changes in PLR induced by mental arithmetic task and cold pressor trials which are often applied in research as model systems to elicit stress response. PLR was recorded before, during and after mental arithmetic and cold pressor tasks in twenty healthy adults (ten males and ten females). Stress-induced sympathetic activation was evident as shown in the increased blood pressure during both tasks. We found that pupillary constriction amplitude and latency did not show significant changes. However, the constriction time and redilation time changed during these tasks. The detailed results have been published in Davis et al. (2013) (attached).

Description of progress in Task #2:

Protocol
There are no previous studies of PLR using functional MRI techniques. As such, it was necessary to adapt the methodology used in previous PLR studies (and Task #1) for use with functional MRI methodology and MRI-safe eye tracking equipment. Data from 7 pilot participants was collected to verify that all components of the combined PLR/ fMRI paradigm provided adequate data quality. We confirmed that a rear-projection system for visual presentation of stimuli provided sufficient luminance to induce PLR in 2 participants. Additional pilot data from 5 participants was collected to verify that PLR parameters could be extracted from data provided by an MRI-compatible video eye-tracking system. Additionally, we confirmed that significant PLR-related changes in brain activity could be detected using the current combined paradigm.

In this paradigm, participants performed a passive viewing task in which they were shown a series of red-filtered, emotionally-neutral images (e.g., landscapes) that changed every 5s to maintain the interest of the participant. Every 20s, the participant was presented a green-filtered light stimulus superimposed over the current image for 100ms. The light stimulus was designed to induce PLR. For each participant, PLR and neural responses were recorded for a total of 96 light stimulus trials. Trials were present over the course of 8 functional MRI runs, each of which lasted approximately 4 ½ minutes.

MRI scans were obtained on a 3T Siemens Trio scanner with a standard 8-channel head coil. For alignment purposes, a set of structural images were collected first using a standard T1-weighted pulse sequence [MP-RAGE sequence: TR = 2400 ms, TE = 3.16 ms, flip angle = 8°, in-plane resolution = 1 x 1 mm, slice thickness = 1 mm, number of slices = 176]. For the PLR functional runs, sets of 38 contiguous axial images (TR = 2500 ms, TE = 30, flip angle = 90°, in-plane resolution = 4.0 x 4.0 mm, slice thickness = 4.0 mm) were acquired parallel to the anterior–posterior commissure plane. This procedure offered whole-brain coverage, including the cerebellum, at a high signal-to-noise ratio.

Participants
In addition to the 7 participants that were run as part of the previously described piloting of the novel PLR/fMRI paradigm, we have collected data from 33 adolescents with ASD and an additional 27 demographically-matched adolescents who were typically developing. All individuals with ASD have met diagnostic criteria on either the Autism Diagnostic Interview-Revised or the Autism Diagnostic Observation Schedule in additional to a clinical diagnosis.
**Current Results**

Data from 16 participants (8 ASD, 8 non-ASD) was omitted from further analysis due to excessive head motion and/or other issues (e.g., unable to complete task). [Note that this rate of data dropout (16 of 60 subjects = 27%) is very typical for MRI research with children and clinical populations.] The remaining dataset comprises of 25 participants with ASD and 19 participants without ASD.

In our initial analysis, we utilized a standard approach to the processing and analysis of the fMRI data (Formisano et al., 2006). As illustrated in the Figure 4 below, the analysis revealed significant PLR-related activation in several visual processing areas including visual cortex and the lateral geniculate nucleus (LGN) in both the ASD and non-ASD groups. We failed, however, to find any significant group-related differences in brain activation that might help to explain the group differences in PLR that we found in previous work (Fan et al., 2009) and Task #1.

![Fig. 4. PLR-related activation shown separately for the non-ASD group (left panel) and ASD group (right panel). Note the activation in the cortical visual areas and also the lateral geniculate nucleus of the thalamus.](image)

Presently we are re-analyzing the fMRI data using a novel, more time-intensive approach that will hopefully allow us to better detect more subtle differences in brain activity. Specifically we are adopting methods that are usually used for resting state fMRI analysis (e.g., Fox et al., 2009) to better help us to account for noise in the MR signal related to subject movement during image acquisition. In most cases when one is conducting a task-related fMRI experiment, the change in brain activation associated with a task is so salient that it is not necessary to do this type of rigorous control for such noise. Basically the signal far outweighs the noise. In the present study, however, the task manipulation (change in luminance) and associated change in brain activation are both subtle. Consequently, this type of approach appears warranted.

In addition, to further test our hypothesis of the involvement of cerebellum, we have undertaken a secondary analysis comparing cerebellum structure between the ASD group and
control group. We recently completed the first step of this process: the manual segmentation of the cerebellum from the skull, dura, and other brain tissue (brainstem, cortex, etc.). Manual segmentation, while time consuming, is considered the gold standard approach in the field.

Looking forward, we anticipate completing the reanalysis of the fMRI data very shortly (within the 1st quarter of Year #4). A manuscript will be prepared shortly thereafter. We also anticipate the results will be presented at an international research meeting (e.g., Annual Meeting of the Cognitive Neuroscience Society, Annual International Meeting for Autism Research) in the Spring 2014.

KEY RESEARCH ACCOMPLISHMENTS:
- We confirmed atypical PLR in children with ASD in a large heterogeneous population;
- We found PLR latency has the potential to track neurodevelopmental trajectory in children;
- Our results suggest autonomic dysfunctions associated with autism;
- We revealed a potential link between PLR and sensory dysfunction in children with autism;
- We tested a total of 67 participants (7 pilot participants; 33 adolescents with ASD; and 27 typically developing adolescents without ASD) in the fMRI part of the study;
- We observed activation in multiple brain regions in association with pupillary light reflex.

REPORTABLE OUTCOMES:

CONCLUSION:
Our current results confirmed atypical PLR and revealed significant different HRV in a large group of children with autism. We found that PLR latency has a potential to be used as a biomarker for normal neurodevelopmental trajectory. In addition, our results revealed that PLR constriction as a physical measure is significantly correlated with sensory behavior in the ASD
group but not in typically developing children. Our fMRI study revealed significant PLR-related activation in several visual processing areas including visual cortex and the lateral geniculate nucleus (LGN) in both the ASD and non-ASD groups. Our current results, however, failed to find any significant group-related differences in brain activation likely due to the small PLR induced effect. We will re-analyze the fMRI data using a more sophisticated approach to further reduce motion related noise so that we may detect more subtle differences in brain activity.

This project serves as an important step to validate and further understand the atypical PLR in autism. As a quick, non-invasive and objective test, PLR can provide quantitative measures of specific neurologic aspects of autism and thereby facilitate our understanding of this complex disorder.

REFERENCES:


APPENDICES: journal publications in 2013 attached.

Atypical Pupillary Light Reflex and Heart Rate Variability in Children with Autism Spectrum Disorder

Chathuri Daluwatte · Judith H. Miles · Shawn E. Christ · David Q. Beversdorf · T. Nicole Takahashi · Gang Yao

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Abstract We investigated pupillary light reflex (PLR) in 152 children with ASD, 116 typically developing (TD) children, and 36 children with non-ASD neurodevelopmental disorders (NDDs). Heart rate variability (HRV) was measured simultaneously to study potential impairments in the autonomic nervous system (ANS) associated with ASD. The results showed that the ASD group had significantly longer PLR latency, reduced relative constriction amplitude, and shorter constriction/redilation time than those of the TD group. Similar atypical PLR parameters were observed in the NDD group. A significant age effect on PLR latency was observed in children younger than 9 years in the TD group, but not in the ASD and NDD groups. Atypical HRV parameters were observed in the ASD and NDD groups. A significant negative correlation existed between the PLR constriction amplitude and average heart rate in children with an ASD, but not in children with typical development.

Keywords Pupillary light reflex · Heart rate variability · Autism · Autonomic nervous system

Introduction

Autism spectrum disorders (ASDs) are complex developmental disorders with symptoms in three core areas: social functioning, communication, and restricted or repetitive behaviors. While much progress has been made regarding ASD, the understanding of its etiology is still evolving (Geschwind and Levitt 2007). Although diagnosis of ASD is based on behavioral assessment, various physical measures have also been used to look for the neurological dysfunctions underlying ASD. Among various measures, pupillary response has been an interesting target. Pupil size is controlled by two antagonistic iris muscles: the sphincter and the dilator (Barbur 2003) and can be easily measured using non-invasive imaging methods. Pupillary responses can reveal a rich set of neurological information (Loewenfeld 1999) and have long been used in both medical practice (Bremner 2009) and psychophysical studies (Laeng et al. 2012).

A few studies compared baseline pupil size in children with ASD and typically developing children, but the results have been inconsistent. Anderson and Colombo (2009) found baseline pupil size was significantly larger in children with ASD than either mental age or chronological age matched controls when they were presented with grey slides. This finding was later replicated in two different samples of children with ASD (Anderson et al. in press). However, Martineau et al. (2011) showed that children with ASD had
significant smaller baseline pupil size than typically developing children in response to a black slide. No difference in baseline pupil size was observed in a study by van Engeland et al. (1991) between the ASD group and typical controls. Fan et al. (2009a) also reported similar baseline pupil size in children with ASD and typically developing children in both dark- and light-adapted conditions although the data variation was significantly higher in the ASD group.

It is recognized in clinical tests (Bremner 2009) that resting pupil size may vary over a wide range even in individuals without any medical problems. On the other hand, the dynamic changes in pupil size induced by various stimuli may provide more reliable information about the neurological system (Bremner 2009). Anderson et al. (2006) reported an atypical pupillary response in children with ASD when viewing children's faces. Specifically, the ASD group showed pupillary constriction in response to children's faces; whereas children with typical development or developmental delays (non-ASD) showed pupil dilation. Martineau et al. (2011) revealed that the pupillary responses to neutral faces, virtual faces, and objects followed a similar three-phase time course in both children with ASD and typical controls, i.e. a rapid initial dilation followed with a rapid constriction and then a slow recovery to baseline. Recently, Wagner et al. (in press) reported that pupillary response to emotional faces was similar in adolescents with ASD and typical controls.

In comparison to the aforementioned social stimuli, luminance change is an easier way to induce consistent pupillary responses (Barbur 2003). When stimulated by a flash of light, pupil undergoes a characteristic process to constrict and then recover (Bremner 2009), which is referred to as pupillary light reflex (PLR). Atypical PLR was previously reported in children with ASD (Rubin 1961; Fan et al. 2009a). Rubin (1961) discovered that the pupillary constriction speed was significantly slower in children with autism than typical controls. Fan et al. (2009a) also reported a significantly longer PLR latency and reduced constriction amplitude associated with ASD.

The PLR pathway includes the retina, pretectal nucleus, Edinger-Westphal nucleus, and ciliary ganglion (Lowenstein and Loewenfeld 1950; Appenzeller 1999). This PLR pathway is largely under the influence of the parasympathetic pathway of the autonomic nervous system (ANS) (Neuhuber and Schrödl 2011). Parasympathetic nerve fibers, which originate in the pupilloconstrictor neurons in the Edinger-Westphal nucleus and synapse at the ciliary ganglion, control the sphincter muscle. Sympathetic nerve fibers from the superior cervical ganglion control the dilator muscle which may also modulate the pupillary constriction process. As a result, PLR parameters can be influenced by ANS dysfunction (Bremner 2009).

ANS dysfunction has been reported in children with ASD in several studies. Ming et al. (2011) reported that families endorsed significantly more symptoms of autonomic dysfunction in their children with ASD than control families. Several studies have reported elevated heart rate in individuals with ASD in comparison to typically developing controls (Kootz and Cohen 1981; Ming et al. 2005; Bal et al. 2010). Ming et al. (2005) also found higher mean arterial and diastolic blood pressure, lower cardiac vagal tone and lower cardiac sensitivity to baroreflex in children with ASD. These findings suggest that children with ASD have an elevated autonomic arousal. In addition, lower baseline respiratory sinus arrhythmia was reported in children with ASD (Bal et al. 2010) suggesting a reduced vagal modulation in ASD. However, Mathewson et al. (2011) demonstrated that baseline cardiac autonomic measures were significantly affected by medication use in adults with ASD.

Heart rate and heart rate variability (HRV), which measures the beat-to-beat variations of the heart rate, are regulated by the ANS. Vagal activity reduces heart rate through the sinoatrial (SA) and atrioventricular (AV) nodes, while sympathetic activation increases the heart rate also through the SA node. HRV parameters are considered an objective assessment of cardiac autonomic function (Kamath and Fallen 1993; Thayer and Sternberg 2006). HRV has been used to evaluate ANS dysfunction in disorders such as panic disorder (Yeragani et al. 1993), schizophrenia (Bär et al. 2007), and sleep disorders (Bonnet and Arand 1998). Interestingly, a significant correlation between HRV and PLR was previously reported in patients with acute schizophrenia (Bär et al. 2008). However, HRV has not been investigated extensively in ASD.

The purpose of this present study is to investigate the atypical PLR associated with ASD in a larger heterogeneous sample. To study the potential association between atypical PLR and other ANS dysfunction in children with ASD, we simultaneously measured HRV during the PLR test. Because of the involvement of cognitive impairment and medication taking in children with ASD, their potential effects on PLR and HRV parameters were studied. We also tested a group of children with non-ASD neurodevelopmental disorders to investigate whether atypical PLR is specific to ASD. Due to the wide age distribution in the test population, the potential age effects on PLR and HRV parameters were also examined.

Methods

Participants

A total of 152 children with an ASD participated in this study (referred to as the “ASD” group). The ages ranged from 5 to
19 years with an average age of 10.7 ± 3.4 years; the group consisted of 135 boys (10.9 ± 3.5 years) and 17 girls (9.8 ± 2.6 years). Of the 152 participants, 145 were patients receiving clinical services at the University of Missouri Thompson Center for Autism and Neurodevelopmental Disorders, an interdisciplinary academic medical center specializing in diagnosis and treatment of ASD. Diagnostic interviews, caregiver questionnaires, and observation focusing on DSM-IV criteria (American Psychiatric Association, 2000) were used for the diagnosis of ASD in these individuals. The Autism Diagnostic Observation Schedule (ADOS) (Lord et al. 1989) was obtained for 112 participants and the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al. 1994) was obtained for 80 patients; the ASD diagnosis was confirmed in all of these cases. Evaluations were conducted by a pediatrician and/or neuropsychologist; if there was disagreement, the results were discussed jointly to reach a consensus diagnosis. The remaining 7 children were diagnosed using a variety of measures, which were reviewed by the authors to confirm the ASD diagnosis. In addition, each of these 7 families completed the Social Communication Questionnaire Lifetime (SCQ) (Eaves et al. 2006) and Social Responsive Scale Questionnaire (SRS) (Constantino and Gruber 2005), all of which were scored above the ASD cutoff.

Among the 152 children with ASD, 86 were diagnosed with classic autism, 32 with Asperger’s Syndrome, and 34 with pervasive developmental disorder—not otherwise specified (PDD-NOS). Seventy children in the ASD group had taken one or more medications (include stimulants, atypical antipsychotics, serotonin reuptake inhibitors, antihistamines, antiepileptics etc.) within 48 h before the PLR test (referred to as the “w/med” group). The remaining children had not taken medication (referred to as the “w/o med” group).

A sample of 116 typically developing healthy children between 6 and 17 years of age without known visual, neurological, or cardiovascular problems comprised a typically developing comparison group (referred to as the “TD” group). Nine children who had a sibling with ASD were excluded from the data analysis. Thus 107 children (mean age = 10.9 ± 2.9 years) were included in the TD group, which consisted of 79 boys (mean age = 11.1 ± 3.1 years) and 28 girls (mean age = 10.6 ± 2.4 years). All participants in the TD group scored below the clinical cutoff (<15) on the Social Communication Questionnaire Lifetime (Eaves et al. 2006) (mean score = 2.3 ± 2.8). None of the TD participants had taken medications within 48 h before the PLR test.

A sample of 36 children ranging in age from 5 to 17 years of age (mean age = 9.9 ± 3.0 years) with intellectual disabilities due to other neurodevelopmental disorders (NDDs) also participated in this study. This group, referred to as the “NDD” group, included 27 boys (mean age = 10.0 ± 3.1 years) and 9 girls (mean age = 9.7 ± 2.6 years). This group included Down syndrome (7), Fragile X syndrome (5), Neurofibromatosis Type One (1), Prader-Willi syndrome (1), and the remainder with idiopathic intellectual impairment. All participants in this group were assessed to confirm that they did not meet the diagnostic criteria for ASD. Nineteen children in the NDD group were on medications similar to those described above for the ASD group.

Intelligence quotient (IQ) scores were available for all participants with the exception of 30 children in the ASD group, 7 in the TD group and 2 in NDD group. The vast majority of IQ scores were derived from the Ravens Progressive Matrices (RPM) (Raven et al. 1996) (n = 81 ASD, 100 TD, and 34 NDD). The remainder were derived from the Wechsler Abbreviated Test of Intelligence (n = 12 ASD), Differential Abilities Scale–2nd Edition (n = 15 ASD), Leiter International Performance Scale–Revised (n = 9 ASD) and Stanford-Binet Intelligence Scales–Fifth Edition (n = 5 ASD). For purposes of later analysis of the relationship between overall intellectual ability and PLR parameters, participants were categorized into either the “Low IQ” group or the “High IQ” group. An IQ equivalent of 80 or higher (9.1 percentile) was used to designate a child with normal-to-above normal intelligence (Wechsler 1991). Thus, the 9.1 percentile was used for those who were assessed with the RPM, and a threshold score of 80 was used for children who had been assessed by other IQ tests. Distributions of the IQ subgroups and medication status of participants are shown in Table 1.

This study was approved by the Institutional Review Board of the University of Missouri. All participants and their legal guardians provided written informed assent and consent prior to participating.

PLR Instrument

The binocular pupillography recording system used in this study is similar to that described previously (Fan et al. 2009a, b). The system uses near-infrared imaging cameras (GC660, Allied Vision Technologies, Stadtroda, Germany) to record pupil images at a speed of 115 frames-per-second (fps). The spatial resolution of the imaging system is 0.035 mm/pixel. A 100 ms optical stimulus is produced using 530 nm green LEDs which illuminates a circular optical diffuser. The illuminated diffuser is positioned at 12.5 cm from the eye and has an effective diameter of 1.27 cm (an equivalent visual field of 5.7°). The stimulus intensity was controlled by adjusting the electric current to the LED and by using different neutral density filters.

To obtain heart rate variability (HRV) in our population, the heart beat signal (RR tachogram) was recorded using a wireless heart rate measuring device (Polar RS800CX,
Polar Electro Oy, Finland). A chest strap with an enclosed heart rate sensor measured the QRS intervals at a rate of 1 kHz. Several studies have found that the performance of this device is consistent with the conventional 12-lead ECG system (Gamelin et al. 2008; Goodie et al. 2000; Nunan et al. 2009; Porto and Junqueira 2009).

Test Procedure

The PLR test procedure was performed as described in detail previously (Daluwatte et al. 2012). In brief, throughout testing the child was seated in a comfortable chair with a back. Heart rate measurements were begun 5 min prior to the PLR testing and continued for 5 min following completion of the PLR testing. Participants fixed the sight on pictures of animals or toys displayed on a dim computer monitor placed 1.3 m away from the eye. PLR was first measured in light adapted (LA) conditions (220 lx room luminance) using 3 different stimulus intensities in ascending order: LA 69.3 cd/m², LA 872.1 cd/m², and LA 8721.1 cd/m². The dark-adapted (DA) PLR was then measured at a stimulation intensity of DA 63.1 cd/m² after 15-min of dark adaptation (<0.01 lx room luminance). For each stimulus condition, the left eye was stimulated 4 times and then the right eye was stimulated 4 times. A 30-sec interval was provided between consecutive stimulations. We tested 43% of the participants in the ASD group, 36% in the TD group, and 53% in the NDD group in the morning, while the remaining was tested in the afternoon.

Data Analysis

The pupilogram was constructed by extracting the pupil size from acquired pupil images as described in detail elsewhere (Fan et al. 2009a). The following PLR parameters were calculated from the pupilogram to quantify the child’s pupillary response (Fig. 1): (1) the baseline pupil diameter (D₀), defined as the average resting pupil diameter before stimulus onset; (2) the relative constriction amplitude, calculated as Aₚ = (D₀² - Dₘ²)/D₀², where Dₘ is the minimal pupil diameter during constriction; (3) the latency (tₗ), defined as the time that elapsed between stimulus onset and the beginning of pupil constriction; (4) the constriction time (t₉), defined as the time interval between the beginning of pupil constriction and when pupil reached minimal diameter Dₘ; (5) the redilation time (tᵣ), calculated as the time interval between the minimal diameter Dₘ and when the pupil recovered to half of the constriction; (6) the constriction velocity (vₖ), calculated as (D₀ - Dₘ)/2t₉; and (7) the redilation velocity (vᵣ), calculated as (D₀ - Dₘ)/4tᵣ. PLR data from both eyes obtained during 8 repeated measurements were averaged to calculate the mean value and standard deviation at each stimulus condition. PLR images of 2 children in the ASD group, 1 child in the TD group, and 3 children in the NDD group could not be processed because of excessive eye movement or closure during the test.

In addition to the average heart rate (AHR), heart rate variability (HRV) was calculated using both time-domain and frequency-domain analyses as explained by Malik (1996). Two time-domain parameters were calculated: (1) the standard deviation of normal to normal (NN) intervals (SDNN) and (2) the root mean square of successive differences (rMSSD). The frequency-domain power spectrum was analyzed using Fast Fourier Transform (FFT).

![Fig. 1 An illustration of the pupilogram and the associated PLR parameters. The optical stimulus is given at time zero. The baseline and minimal pupil diameters are calculated as D₀ = 2√A₀/π and Dₘ = 2√Aₘ/π, respectively. The relative constriction amplitude is obtained as Aₚ = (A₀ - Aₘ)/A₀. The constriction and redilation velocities are calculated as vₖ = (D₀ - Dₘ)/2t₉ and vᵣ = (D₀ - Dₘ)/4tᵣ, respectively](image-url)
frequency-domain HRV parameters were calculated: the normalized power of the high-frequency band (HFₚ) (HF = 0.15–0.4 Hz) and the LF/HF power ratio, where the low-frequency bandwidth was 0.04–0.15 Hz. HFₚ is generally considered as an indicator of vagal activity and is correlated with rMSSD (Malik 1996). SDNN carries influences from both parasympathetic and sympathetic modulation (Malik 1996). The LF/HF ratio may reflect the “sympathetic outflow” or the “sympathovagal balance” (Malik 1996; Berntson et al. 1997).

To determine any potential effect of participating in the PLR procedure on heart rate variability, the HRV was analyzed in the following five different “HRV measurement phases”: (1) before the PLR test (5 min), (2) during LA PLR (10 min), (3) during dark adaptation (15 min), (4) during DA PLR (5 min), and (5) after the PLR test (5 min). We were not able to acquire HRV in 9 children in the ASD group, 1 in the TD group, and 1 in the NDD group because the participants declined to wear the heart rate sensor. A malfunction of the heart rate sensor resulted in missing HRV data in 2 other children in the ASD group.

The Kolmogorov–Smirnov test was used to verify normal distributions of all measured PLR and HRV parameters. For each PLR and HRV parameter, the Analysis of Covariance (ANCOVA) using the PROC MIXED procedure in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA) was applied to examine the effects of group (TD, ASD, and NDD), age, and test conditions (stimulus intensity/HRV measurement phase and time of day of the test). Follow up analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), and t-tests with Bonferroni correction were used appropriately to confirm effects revealed by the ANCOVA model. ANOVA model was applied to study the effects of IQ (High IQ and Low IQ) and medication (“w/o med” and “w/med”) in the ASD and NDD groups, and the effect of ASD diagnosis (classic autism, Asperger’s, and PDD-NOS) in the ASD group. The method reported by Steyn and Ellis (2009) was applied to evaluate effect size (̂νⱼ) for group differences using MANOVA. An ̂νⱼ value of 0.02, 0.13 and 0.26 was considered as a small, medium and large effect, respectively (Steyn and Ellis 2009). Pearson product moment correlation was applied to study correlation between PLR parameters and HRV parameters. A p value < 0.05 was considered significant.

Results

The mean and standard deviations of all measured PLR and HRV parameters in the TD, ASD, and NDD groups are shown in Tables 2 and 3, respectively.

The ANCOVA model revealed that the stimulation condition (adaptation and stimulus intensity) had a statistically significant effect (p < 0.0001) on all PLR parameters, including the constriction time (t½), relative constriction amplitude (ΔA₀), latency (tᵢ), redilation time (tᵣ), constriction velocity (v_c), and redilation velocity (v_r). As expected, the resting pupil size was larger in DA than in LA. The PLR constriction amplitude, constriction time, and redilation time all increased with stimulus intensity, whereas the PLR latency decreased with stimulus intensity at the same adaptation. The constriction and redilation velocities also increased with stimulus intensity in LA and were larger in DA tests than LA tests at similar stimulus intensities. The interaction between group and stimulus was not significant for any of the PLR parameters, which suggests that the stimulus dependency was similar in all subject groups.

Subject Group Differences

Group Differences in PLR Parameters

The PLR parameters were significantly different between the TD and ASD groups, and between the TD and NDD groups, but not between the ASD and NDD groups.

The ANCOVA model indicated that the group (TD, ASD, and NDD) had a significant effect on PLR latency (F₂,₁₁₀₇ = 150.44 p < 0.0001), relative constriction amplitude (F₂,₁₁₀₆ = 29.96 p < 0.0001), constriction time (F₂,₁₁₀₃ = 31.69 p < 0.0001), and redilation time (F₂,₁₀₉₆ = 14.67 p < 0.0001). Post-hoc MANOVA confirmed that the ASD and NDD groups had a significantly longer latency (F₄,₂₂₉ = 23.24 p < 0.0001 ̂ν₂ = 0.28 for ASD; F₄,₁₃₀ = 21.69 p < 0.0001 ̂ν₂ = 0.38 for NDD) and lesser relative constriction amplitude (F₄,₂₃₁ = 4.47 p = 0.002 ̂ν₂ = 0.06 for ASD; F₄,₁₃₀ = 3.74 p = 0.007 ̂ν₂ = 0.08 for NDD) than those of the TD group for all testing conditions. The ASD group also had a shorter constriction time (MANOVA F₄,₁₁₀₃ = 31.69 p < 0.0001) and relative constriction amplitude (F₄,₂₃₁ = 4.47 p = 0.002 ̂ν₂ = 0.06) and redilation time (MANOVA F₄,₂₂₅ = 3.39 p = 0.01 ̂ν₂ = 0.04) than those of the TD group. The mean PLR latency of the NDD group appeared to be longer than that of the ASD group, but the difference was not statistically significant (MANOVA F₄,₁₅₂ = 1.71 p = 0.15). No significant group differences were found for other PLR parameters.

Group Differences in AHR and HRV Parameters

The ASD and NDD groups had significantly different AHR and HRV parameters than the TD group. The NDD group showed a significantly faster AHR than the ASD group.

The ANCOVA model revealed a significant group effect on AHR (F₂,₁₃₄₃ = 50.81 p < 0.0001) and on time-domain
Table 2 Summary of PLR results

<table>
<thead>
<tr>
<th>Stimulus intensity (cd/m²)</th>
<th>Resting pupil diameter (mm)</th>
<th>PLR latency (ms)*</th>
<th>Constriction (%)*</th>
<th>Constriction time (ms)*</th>
<th>Redilatation time (ms)*</th>
<th>Constriction velocity (mm²/s)</th>
<th>Redilatation velocity (mm²/s)</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>LA 69.3</td>
<td>6.58 ± 0.61</td>
<td>274.3 ± 23.9</td>
<td>11.8 ± 5.5</td>
<td>370.7 ± 73.4</td>
<td>402.0 ± 86.3</td>
<td>0.81 ± 0.44</td>
<td>0.37 ± 0.20</td>
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<tr>
<td>LA 872.1</td>
<td></td>
<td>239.0 ± 16.2</td>
<td>26.9 ± 7.1</td>
<td>399.2 ± 52.8</td>
<td>498.1 ± 99.9</td>
<td>1.75 ± 0.75</td>
<td>0.72 ± 0.30</td>
</tr>
<tr>
<td>LA 8721.1</td>
<td></td>
<td>214.4 ± 14.4</td>
<td>40.8 ± 7.2</td>
<td>464.5 ± 51.2</td>
<td>595.6 ± 116.6</td>
<td>2.37 ± 0.95</td>
<td>0.98 ± 0.45</td>
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<tr>
<td>DA 63.1</td>
<td>7.44 ± 0.77</td>
<td>244.0 ± 15.4</td>
<td>44.7 ± 6.9</td>
<td>580.2 ± 59.4</td>
<td>804.4 ± 171.8</td>
<td>2.41 ± 0.97</td>
<td>0.91 ± 0.36</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LA 69.3</td>
<td>6.50 ± 0.81</td>
<td>302.2 ± 32.2</td>
<td>9.6 ± 6.1</td>
<td>336.7 ± 75.6</td>
<td>370.3 ± 89.2</td>
<td>0.76 ± 0.50</td>
<td>0.35 ± 0.21</td>
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<tr>
<td>LA 872.1</td>
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<td>265.3 ± 25.4</td>
<td>22.5 ± 8.3</td>
<td>364.5 ± 63.4</td>
<td>455.8 ± 104.0</td>
<td>1.65 ± 0.76</td>
<td>0.68 ± 0.31</td>
</tr>
<tr>
<td>LA 8721.1</td>
<td></td>
<td>237.7 ± 22.5</td>
<td>36.6 ± 8.5</td>
<td>434.1 ± 62.9</td>
<td>560.7 ± 106.1</td>
<td>2.39 ± 0.91</td>
<td>0.95 ± 0.35</td>
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<tr>
<td>DA 63.1</td>
<td>7.47 ± 0.88</td>
<td>262.7 ± 18.5</td>
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<td>552.1 ± 73.8</td>
<td>737.8 ± 155.0</td>
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<td>LA 69.3</td>
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<td>372.9 ± 77.1</td>
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<tr>
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<td>566.3 ± 107.4</td>
<td>730.0 ± 116.5</td>
<td>2.00 ± 0.85</td>
<td>0.79 ± 0.35</td>
</tr>
</tbody>
</table>

The results are represented as group mean ± standard deviation
* Significant group difference (ANCOVA p < 0.0001)

Table 3 Summary of HRV results

<table>
<thead>
<tr>
<th>HRV measurement phase</th>
<th>AHR (bmp)*</th>
<th>SDNN (ms)*</th>
<th>rMSSD (ms)*</th>
<th>LF/HF (n.u.)</th>
<th>HF (%)</th>
</tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>90.1 ± 12.4</td>
<td>64.2 ± 24.3</td>
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<td>31.2 ± 10.3</td>
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<tr>
<td>2</td>
<td>90.0 ± 12.1</td>
<td>69.7 ± 24.3</td>
<td>36.9 ± 17.7</td>
<td>4.5 ± 5.2</td>
<td>22.8 ± 8.0</td>
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<tr>
<td>3</td>
<td>93.1 ± 12.5</td>
<td>66.4 ± 28.0</td>
<td>32.6 ± 17.1</td>
<td>3.4 ± 1.6</td>
<td>26.3 ± 10.0</td>
</tr>
<tr>
<td>4</td>
<td>91.0 ± 13.0</td>
<td>72.0 ± 26.3</td>
<td>36.4 ± 18.2</td>
<td>4.4 ± 3.0</td>
<td>22.7 ± 9.5</td>
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<td>5</td>
<td>92.9 ± 13.3</td>
<td>65.4 ± 27.2</td>
<td>33.2 ± 17.7</td>
<td>4.1 ± 0.3</td>
<td>25.2 ± 10.5</td>
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<tr>
<td>ASD</td>
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<td></td>
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<tr>
<td>1</td>
<td>95.2 ± 14.0</td>
<td>56.9 ± 20.1</td>
<td>32.2 ± 15.6</td>
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<td>29.5 ± 10.8</td>
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<tr>
<td>2</td>
<td>96.1 ± 13.7</td>
<td>61.1 ± 20.9</td>
<td>31.4 ± 15.6</td>
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<td>3</td>
<td>99.8 ± 12.9</td>
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<td>27.4 ± 14.0</td>
<td>3.4 ± 1.7</td>
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<td>4</td>
<td>97.0 ± 13.0</td>
<td>60.6 ± 23.8</td>
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<td>101.5 ± 13.8</td>
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<td>104.7 ± 14.5</td>
<td>45.9 ± 15.4</td>
<td>23.3 ± 12.3</td>
<td>3.5 ± 1.8</td>
<td>25.5 ± 9.4</td>
</tr>
</tbody>
</table>

The results are represented as group mean ± standard deviation. The HRV measurement phases are numbered as 1 before PLR test, 2 during LA PLR, 3 during dark adaptation, 4 during DA PLR, and 5 after PLR test
bpm beats per minute, SDNN standard deviation of normal to normal (NN) intervals, rMSSD root mean square of successive differences, LF low frequency, HF high frequency
* Significant group difference (ANCOVA p < 0.0001)

HRV parameters (F₂,1340 = 41.92 p < 0.0001; and F₂,1340 = 27.46 p < 0.0001 for SDNN and rMSSD respectively). Post-hoc MANOVA confirmed that children with ASD had a significantly faster heart rate than that of typical controls in all 5 HRV measurement phases (F₅,218 = 3.32 p = 0.007 r² = 0.05) (Table 3). The mean values of SDNN...
and rMSSD were lower in the ASD group than the TD group. However, MANOVA revealed that these differences were not statistically significant (F_{5,217} = 2.00, p = 0.08; and F_{5,217} = 1.46, p = 0.20 for SDNN and rMSSD, respectively). The AHR of the NDD group was significantly faster than that of the ASD group (MANOVA F_{5,146} = 2.63, p = 0.03, \tilde{\eta}^2_{A,r=1} = 0.05). The NDD group also had a significantly faster AHR (F_{5,132} = 5.41, p = 0.0001, \tilde{\eta}^2_{A,r=1} = 0.14), lower SDNN (F_{5,131} = 4.70, p = 0.0006, \tilde{\eta}^2_{A,r=1} = 0.12) and lower rMSSD (F_{5,131} = 2.63, p = 0.03, \tilde{\eta}^2_{A,r=1} = 0.06) than those of the TD group.

Age Effect

A significant age effect on PLR latency was observed in the TD group, but not in the ASD group. Both ASD and TD groups showed similar age trends for average heart rate and HRV parameters.

In the TD group, the PLR latency decreased from 6 to 8 years and reached a plateau thereafter (Fig. 2). One 16-year-old and two 13-year-olds in the TD group were identified as outliers on the regression line (PROC ROBUSTREG procedure in SAS); hence, their data were not included in the data shown in Fig. 2. In children 6–8 years of age, the ANCOVA model indicated that the Age*Group interaction was a significant factor on PLR latency (F_{4,265} = 3.26, p = 0.01), suggesting that PLR latency had different age profiles in the 3 subject groups. Analysis using the CONTRAST statement of GLM procedure with matrix \[ [+1,0,-1] \] in SAS confirmed that latency decreased from 6 to 8 years in the TD group (F_{1,21} = 0.22, p = 0.64; F_{1,21} = 4.85, p = 0.039; F_{1,21} = 8.97, p = 0.007; and F_{1,21} = 7.49, p = 0.012 for latency measured at LA 69.3 cd/m², LA 872.1 cd/m², LA 8721.1 cd/m², and DA 63.1 cd/m², respectively). However, this decreasing trend did not exist in the ASD group at any of the 4 stimulus intensities (F_{1,36} = 0.55, F_{1,35} = 0.79; F_{1,37} = 0.93; and F_{1,35} = 0.81).

For further confirmation, the lines in Fig. 2 show the best curve fitting results using an exponential decay function \[ y = a \exp(-bx) + c \] with the curve-fitting tool in Matlab (Mathworks, MA). The TD results were well fitted with this function, with R² ranging from 0.56 to 0.88. However, either the ASD results could not be fitted with this exponential decay function or the decay was much slower than the TD results. The age effect was also not significant in the NDD group, although the number of participants was much smaller.

The ANCOVA model revealed a significant age effect on AHR and both time- and frequency-domain HRV parameters. The AHR, SDNN, and HFN values measured during HRV measurement phase 1 (before the PLR test) in the TD and ASD groups are shown in Fig. 3. The AHR decreased with age in both groups. SDNN showed little change before 12 years of age but was increased in older children. HFN decreased with age in both the TD and ASD groups. Similar results were obtained in the other HRV measurement phases. A similar age effect on AHR was observed in the NDD group, but the time domain and the
frequency domain parameters did not show a significant age effect in this group.

Medication Effect

A medication effect was observed on average heart rate and HRV parameters, but not on PLR parameters.

The PLR latency in the TD group was significantly different from that in both the “w/med” ASD group ($F_{4,159} = 20.35$ $p < 0.0001$) and the “w/o med” ASD group ($F_{4,171} = 15.80$ $p < 0.0001$). The TD group also had significantly larger PLR constriction than both the “w/med” ASD group ($F_{4,160} = 3.84$ $p = 0.005$) and the “w/o med” ASD group ($F_{4,172} = 2.90$ $p = 0.023$). Similarly, the PLR constriction time was significantly longer in the TD group than in both the “w/med” ASD group ($F_{4,159} = 5.56$ $p = 0.0003$) and the “w/o med” ASD group ($F_{4,170} = 2.77$ $p = 0.029$). Though the “w/med” ASD group appeared to have a slightly greater PLR latency, lesser constriction amplitude, and shorter constriction time than those of the “w/o med” ASD group (Fig. 4), the MANOVA indicated that these differences were not significant ($F_{4,123} = 0.92$ $p = 0.45$; $F_{4,125} = 0.64$ $p = 0.64$; and $F_{4,122} = 1.25$ $p = 0.29$ for latency, constriction amplitude, and constriction time respectively). The redilation time was different only between the TD and “w/med” ASD groups ($F_{4,158} = 3.63$ $p = 0.007$) but not between the TD and “w/o med” ASD group ($F_{4,168} = 1.87$ $p = 0.11$) or between the “w/med” and “w/o med” ASD groups ($F_{4,119} = 0.96$ $p = 0.43$).

The ASD “w/med” group had faster AHR and lesser SDNN and rMSSD than those of the ASD “w/o med” group (Fig. 5). The MANOVA test indicated significant group differences between the TD and ASD “w/med” group with respect to average heart rate ($F_{5,157} = 3.75$ $p = 0.003$), SDNN ($F_{5,156} = 2.23$ $p = 0.066$) and rMSSD ($F_{5,156} = 2.53$ $p = 0.031$). However, these parameters were not significantly different between the TD and ASD “w/o med” groups or between “w/med” and “w/o med” ASD groups. Similar results between the “w/med” and “w/o med” groups were obtained in the NDD group.

IQ Effect

No significant IQ effect was observed on any PLR or HRV parameters in the ASD group. Children with ASD and a “Low IQ” had a slightly longer latency, lesser constriction amplitude, shorter constriction/redilation times, and smaller pupil diameter than those with a “High IQ” (Fig. 6). However, the MANOVA model indicated that the differences between the “High IQ” and “Low IQ” groups were only marginally significant with respect to PLR latency ($F_{4,98} = 2.28$ $p = 0.066$) and not significant for constriction amplitude ($F_{4,100} = 0.37$ $p = 0.83$), constriction time ($F_{4,97} = 1.84$ $p = 0.13$), and redilation time ($F_{4,95} = 0.86$ $p = 0.49$). The TD group had significantly longer latency, lesser constriction amplitude, and shorter constriction time than both the “High IQ” and “Low IQ” ASD groups.

Children with ASD and a “Low IQ” had a slower mean AHR, larger SDNN and rMSSD than those with a “High
IQ” (Fig. 7). However, the MANOVA model indicated that the differences between the “High IQ” and “Low IQ” groups were insignificant ($F_{5,88} = 0.93, p = 0.47$ for AHR; $F_{5,88} = 0.51, p = 0.77$ for SDNN; and $F_{5,88} = 0.66, p = 0.66$ for rMSSD). The TD group had significantly slower AHR than the “High IQ” ASD group ($F_{5,167} = 4.21, p = 0.001$), but not the “Low IQ” ASD group ($F_{5,137} = 1.72, p = 0.13$). An IQ effect was not found for any other PLR and HRV parameters in the ASD group. Similar results were observed in the NDD group.

Interaction Between IQ and Medication

The interaction between IQ and medication appeared to have a significant effect on PLR latency in the ASD group as revealed by ANOVA ($F_{1,453} = 12.74, p = 0.0004$) (Fig. 8). Children in the “High IQ” group did not show a difference with medication (MANOVA $F_{4,87} = 0.34, p = 0.85$). In the “Low IQ” group, those using medication appeared to have a longer latency than those who were not using medication. However, this difference did not reach statistical significance in the MANOVA test ($F_{4,43} = 1.40, p = 0.25$).

Further analysis in the “w/o med” subgroups indicated that the “High IQ” group had a similar latency as the “Low IQ” group (MANOVA $F_{4,53} = 0.87, p = 0.5$). However, the IQ effect was significant in the “w/med” group at the highest stimulus intensity of LA 8721.1 cd/m$^2$ ($t$ test $p = 0.03$, Bonferroni corrected) with the “Low IQ” showing a longer latency than the “High IQ” group.

The above interaction effect was not significant on other PLR parameters or on any HRV parameters in the ASD group. In addition, the above interactions were not significant in the NDD group.

Effects of PLR Test on HRV

The HF$_N$ and LF/HF parameters changed significantly when transitioning between resting periods and PLR testing periods in all 3 groups. Such changes were smaller in the ASD and NDD groups than the TD group.

The ANCOVA model indicated that the HRV measurement phase had a statistically significant effect on AHR, SDNN, rMSSD, LF/HF, and normalized HF power ($F_{4,1343} = 6.29$, $p < 0.0001$; $F_{4,1340} = 4.29$, $p = 0.01$; $F_{4,1340} = 4.89$, $p = 0.006$; $F_{4,1340} = 8.84$, $p < 0.0001$; and $F_{4,1320} = 18.91$, $p < 0.0001$, respectively). The interaction between group and HRV measurement phase was not significant. However, post hoc one-way ANOVA indicated that the HRV measurement phase effect was significant only for the LF/HF ($p < 0.013$) and HF$_N$ ($p < 0.005$) in all 3 subject groups.

The changes of the 2 frequency domain parameters between 2 adjacent HRV measurement phases are shown in...
HF decreased when transiting from resting phases to test phases (phase 1–2 and phase 3–4) and increased when transiting from test phases to resting phases (phase 2–3 and phase 4–5). The changes in the LF/HF parameters were opposite of those observed in HF. The HF changes were significantly larger in the TD group than in the ASD groups (MANOVA $F_{4,226} = 4.81 \ p = 0.001$). However, the LF/HF ratio changes between the TD group and the ASD group was not significantly different (MANOVA $F_{4,231} = 1.73 \ p = 0.14$). The above changes were not significantly different between the ASD and NDD groups (MANOVA $F_{4,157} = 0.81 \ p = 0.52$; and $F_{4,160} = 0.99 \ p = 0.42$ for HF changes and LF/HF ratio changes, respectively).

**Correlation Between PLR and HRV**

PLR constriction amplitude was significantly correlated with average heart rate in the ASD group in all LA tests ($r = -0.3, \ p < 0.01$) (Fig. 10). This correlation was observed in both the “w/o med” ASD and “w/med” ASD groups. However, this correlation was not observed in typically developing children ($p > 0.05$). This correlation was significant in the NDD group only at the highest stimulus intensity of LA 872.1 cd/m². Correlations were not found between other PLR and HRV parameters.

**Subject Group Discrimination**

Using the DISCRIM procedure in SAS, a step-wise (PROC STEPDISC) variable selection procedure was used to identify the best candidate parameters to discriminate between the ASD and TD groups. With a significance level of $p = 0.15$, the procedure selected following measurements for the discrimination model: latency at LA 69.3 cd/m² and LA 8721.1 cd/m², constriction amplitude at LA 69.3 cd/m² and LA 8721.1 cd/m², constriction time at LA 69.3 cd/m² and LA 872.1 cd/m²; and resting pupil diameter at DA. The discriminant analysis results were significant ($\chi^2(28) = 85.5, \ p < 0.0001$) with 81.5 % subjects successfully classified (23.6 % false negatives and 12.3 % false positives). When the NDD group was included in the test data set, 72.4 % of them were classified into the ASD group and 27.6 % were classified into the TD group. Notably, the majority (53.8 %) of the misclassified children with typical development were female although females comprised only a small portion of the overall sample. A slightly higher successful discrimination rate (83.4 %) was obtained when the DISCRIM procedure was applied to the dataset after removing all female participants, with a 21.7 % false-negative rate and a 9.0 % false-positive rate. Examination of autism specific variables revealed that 9.3 % children with classic autism were misclassified, along with 25 % with Asperger’s and 26.5 % with PDD-NOS. Of the children with ASD who were misclassified, 76.7 % were in the “High IQ” group.
The current results confirmed the previous observation by Fan et al. (2009a) that children with an ASD had longer latency and less relative constriction than children with typical development. Furthermore, we found that the constriction time and redilation time were shorter in children with an ASD compared to children with typical development. Due to the predominance of male participants in this study, we also analyzed the data with only the male participants, and all group differences remained the same. Our analyses did not show a significant difference between the PLR and HRV measurements obtained in the mornings and those obtained in the afternoons. We did not find any ASD diagnosis (classic autism, Asperger’s Syndrome, and PDD-NOS) effects on PLR and HRV measurements.

It is interesting that the age trend of PLR latency observed in typically developing children was not observed in the ASD group. It is important to note that this trend (Fig. 2) is in sharp contrast to the age profiles of AHR and HRV (Fig. 3) which are similar in both TD and ASD groups. As an additional comparison, the age trend of PLR latency in typical controls is different from the maturation of the visual system characterized by pattern visual evoked potential (VEP), which stabilizes after 6 months of life (McCulloch and Skarf 1991), but is similar to the trend observed in flash VEP (Dockstader et al. 2012). In addition, this age trend is coincident with the white matter maturation trend revealed in diffuse-tensor MRI studies (Bashat et al. 2007). It has been reported that children with an ASD have accelerated white matter maturation before 4 years of age (Bashat et al. 2007; Weinstein et al. 2011), but this trend is reversed after 4 years of age (Vissers et al. 2012). This appears to be consistent with our observation on PLR latency (Fig. 2).

The ASD group showed a faster average heart rate than that of the typically developing controls, which is similar to previous findings (Palkovitz and Wiesenfeld 1980; Kootz and Cohen 1981; Ming et al. 2005; Bal et al. 2010). The faster average heart rate suggests an increased sympathetic tone or/and impaired parasympathetic control in children with an ASD. The study by Levy (1990) suggested that resting heart rate is predominantly controlled by vagal

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**Fig. 6** The IQ effects on a PLR latency, b constriction amplitude, c constriction time and d redilation time in the ASD group. The error bars indicate the standard error.
modulation. The ASD group also had smaller PLR constriction amplitude, indicating lower parasympathetic modulation (Barbur 2003; Clarke 2007). A previous cardiovascular study showed that children with an ASD had lower parasympathetic activity (Ming et al. 2005). Interestingly, a statistically significant negative correlation existed between PLR constriction and average heart rate in the ASD group but not in the typically developing children. This observed correlation may indicate possible parasympathetic dysregulation associated with ASD. Significant correlations between PLR and HRV parameters were also previously reported in adults with acute schizophrenia (Bär et al. 2008), but an unequivocal correlation was not found in healthy adults (Bär et al. 2009) or healthy children (Daluwatte et al. 2012).

Frequency-domain HRV parameters appeared to change significantly when transiting between the rest and test phases in both the ASD and TD groups. Specifically, the $H_{FN}$ decreased during transition from a resting phase to a PLR test phase (1–2 and 3–4) and increased during transition from a testing phase to a resting phase (2–3 and 4–5). The LF/HF showed a reversed trend. This observation is similar to the previously reported posture-induced HRV changes associated with orthostatic stress (Mukai and Hayano 1995; Montano et al. 1994; Yeragani et al. 1993). The PLR test requires the participant to incline slightly forward ($\sim 15^\circ$), and this posture change can cause elevation in sympathetic tone due to muscle stress. Delaney and Brodie (2000) reported that psychological stress can increase low-frequency HRV while decreasing high-frequency HRV. Nevertheless, the observation of significantly smaller PLR test-related HRV changes in the ASD group suggested less variability in vagal and sympathetic modulation in this population. This is similar to the results reported by Toichi and Kamio (2003), who found that typical controls showed a significant decrease in cardiac autonomic function during a mental arithmetic task while the ASD group did not show significant changes. The observation in the ASD group was not caused by medication because the conclusion remained the same with only the “w/o med” ASD group used in the data analysis.

The current results did not support a significant IQ effect on PLR parameters. The apparent IQ effect on PLR latency was complicated by the medication effects. The analysis of the interaction between IQ and medication supported the notion that IQ alone does not have a significant effect on PLR latency. In the “w/o med” ASD group, where the medication effect was excluded, those in the “High IQ” group showed similar latencies as those in the “Low IQ” group. Medication effect was not observed in the “High IQ” group; however, in the “Low IQ” ASD group, latency tended to be greater in children using medication than in those not using medication. Children in the “Low IQ” group may have required medications for their severe symptoms. In other words, the observed longer PLR latency in this group of participants (“Low IQ” and “w/med”) may have been associated with their symptoms rather than with medication. Similar effect of IQ and medication interaction.
was not observed in other PLR and HRV parameters. A trend of medication effects was observed in the results especially on average heart rate and time-domain HRV parameters. However, the difference between “w/o med” and “w/med” ASD groups did not reach statistical significance. Most of the children in the “w/med group” were taking multiple medications, which made it difficult to clarify the effect of individual medication. This observation requires further investigation.

The discrimination analysis results reported herein were not as robust as those reported by Fan et al. (2009a); this was mostly likely due to the increased sample size and the heterogeneity therein. The different age trends in the ASD and TD groups strongly suggested that age should be considered when interpreting PLR measurements. Despite the low number of participants in the non-ASD NDD group, our results indicated that the NDD group had similar PLR and HRV parameters as those of the ASD group. Therefore, the observed atypical PLR parameters were not specific to ASD. In other words, the same dysfunctions involved in the PLR pathway are mostly likely implicated in both ASD and other neurodevelopmental disorders.

Fig. 8 The effect of IQ and medication interaction on PLR latency at stimulation intensities of a LA69.3 cd/m², b LA872.1 cd/m² c LA8721.1 cd/m², and d DA63.1 cd/m² in the ASD group. *t test p = 0.03, Bonferroni corrected

Fig. 9 The change of frequency domain HRV parameters between consecutive HRV measurement phases. a HF normalized power and b LF/HF ratio. The error bars indicate the standard error. The HRV measurement phases are numbered as 1 before PLR test, 2 during LA PLR, 3 during dark adaptation, 4 during DA PLR and 5 after PLR test
Fig. 10 The correlation between average heart rate and relative constriction amplitude in a children with ASD and b typical controls. The data shown were measured at stimulus intensity of LA 872.1 cd/m². (Pearson’s $r = -0.3^*, -0.3^{**}, -0.3^{***}, -0.1$ in the ASD group and $r = -0.06^*, -0.1^*, -0.1^*, -0.02^*$ in the TD group at stimulus LA 69.3 cd/m², LA 872.1 cd/m², LA 8721.1 cd/m², and DA 63.1 cd/m², respectively. **$p < 0.001$, *$p < 0.01$, *$p > 0.05$)

Conclusion

We measured PLR and HRV simultaneously in a large heterogeneous group of children with an ASD, age-matched typically developing children, and children with an NDD other than an ASD. Children with an ASD or NDD showed atypical PLR, including greater latency, less constriction amplitude, and shorter constriction/redilation times. We also found a significant age effect in children with typical development that was not observed in children with an ASD; this may be due to altered brain development associated with ASD. Furthermore, we found a correlation between PLR and HRV parameters in the ASD group; this correlation was absent in children with typical development. These findings, in addition to atypical PLR profiles, suggest that an abnormality in the ANS is associated with ASD. The similar atypical PLR observed in ASD and NDD indicates that PLR differences are implicated in a wide range of neurodevelopmental disorders. As a simple and economic neurological test, PLR may be potentially useful for early screening of neurodevelopmental disorders in children.

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Effects of cold-pressor and mental arithmetic on pupillary light reflex

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Effects of cold-pressor and mental arithmetic on pupillary light reflex

B C Davis, C Daluwatte, N C Colona and D G Yao

Department of Biological Engineering, University of Missouri, Columbia, MO 65211, USA
E-mail: YaoG@missouri.edu

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Abstract
Dynamic pupillary light reflex (PLR) is a simple neurological test that can be useful for assessment of autonomic disorders. In this study, we investigated the changes in PLR induced by mental arithmetic task and cold pressor trials which are often applied in research as model systems to elicit autonomic responses. PLR was recorded before, during and after mental arithmetic and cold pressor tasks in 20 healthy adults (ten males and ten females). Stress-induced sympathetic activation was evident as shown in the increased blood pressure during both tasks. Although the pupillary constriction amplitude did not show significant changes, both constriction time and redilation time changed during the tasks. A significant gender effect was observed in cold pressor that suggested more sympathetic activation in males and faster parasympathetic activation in females in response to light stimulation under cold pressor.

Keywords: pupillary light reflex, autonomic nervous system, stress
(Some figures may appear in colour only in the online journal)

1. Introduction

Pupil size is controlled by the antagonistic dilator and sphincter muscles in the iris (Barbur 2004). The sphincter is innervated primarily by the parasympathetic nervous system and its contraction leads to pupil constriction. The dilator mediates pupillary dilation and is innervated primarily by the sympathetic nervous system. Pupil size undergoes a characteristic change under a sudden increase in retinal luminance: an initial rapid constriction followed with a slow redilation. Such pupillary response is referred to as pupillary light reflex (PLR). Autonomic nervous system (ANS) modulation is evident in PLR responses (Tavernor et al 2000) and the dynamic PLR parameters are considered useful for reliable ANS assessment (Bremner 2009). PLR parameters linked to the constriction phase such as the constriction amplitude and constriction time fall under parasympathetic control; whereas base pupil radius and PLR parameters measured in the redilation phase such as the redilation time are mainly governed by the sympathetic nervous system (Keivanidou et al 2010).
PLR has been found to be affected by stress and anxiety (Bakes et al. 1990, Bitsios et al. 2002). Bakes et al. (1990) reported that the PLR constriction was smaller in patients with anxiety disorder. Bitsios et al. (2002) found that the threat-induced anxiety reduced the PLR constriction amplitude and a negative correlation existed between state anxiety level and PLR constriction. In addition, threat also increased the initial pupil diameter. Interestingly, two frequently applied laboratory stressors: mental stress (Yamanaka and Kawakami 2009) and cold pressor (CP) stress (Tavernor et al. 2000) were also shown to cause autonomic nervous system-mediated pupil dilation. Mental arithmetic (MA) requires subjects to solve a series of arithmetic problems, which was shown to increase sympathetic activity (Freeman 2006, Liu et al. 2011) and inhibit parasympathetic activity (Sloan et al. 1991). Increases in blood pressure (Willemsen et al. 2000) and marginal increases in heart rate (HR) (Tanida et al. 2004) have been reported. The CP test involves submerging the subject’s hand up to mid-forearm in a bath of ice water for 60–120 s. CP induces pain and emotional distress which produce sympathetic activation with measurable increases in HR and blood pressure (Zygmun and Stanczyk 2010).

The effects of mental stress and CP on resting pupil size have been documented previously (Yamanaka and Kawakami 2009, Tavernor et al. 2000). In addition, Steinhauer et al. (2000) found that an arithmetic task changed multiple dynamic PLR responses. However, the effect of CP on dynamic PLR responses is largely unknown. In this study, we compared changes in PLR induced by mental stress and CP in healthy adult volunteers. We also examined the gender differences in dynamic pupil change, HR, and blood pressure during CP and mental stress tests. With this study we hope to gain a better understanding of the different autonomic components of the PLR pathway, and how these components are affected by different types of stress.

2. Methods

2.1. Subjects

Twenty volunteers (age 18–21), ten males (20.4 ± 0.8 years old) and ten females (19.9 ± 0.9 years old) were recruited from the student population at the University of Missouri-Columbia. All were in good health and had no history of eye-related disorders. Before the experiment, each subject completed the state-trait anxiety inventory for adults (Spielberger et al. 1970). Subject anxiety levels were examined based on their current state and enduring personality traits.

The study was approved by the university IRB board, and all subjects gave informed consent prior to participating in the experiment.

2.2. PLR measurements

PLR was measured using a two-channel pupillographic system (Daluwatte et al. 2012). During the test, participants looked at a screen 0.6 m away through a view port in the system. The screen was covered with a dark red film to avoid affecting pupil size. The 0.1 s stimulation light was produced by green LEDs at 530 nm. The stimulation light had an intensity of $3.3 \times 10^{-5}$ W m$^{-2}$ and a field size of $5.3^\circ$ measured at the position of the eye, leading to a luminous intensity of 0.74 cd m$^{-2}$. All tests were conducted in the morning between 9:30 am and 12:10 pm in a light adapted condition with a room illumination of 255 Lux.

The stimulus light was applied to the right eye in odd numbered trials and to the left eye in even numbered trials during the MA task. Due to the time limit in the CP task, only right eyes were stimulated. The image sequences of both pupils were recorded on CCD cameras.
(119 fps) for the entirety of each measurement. The stimulus was presented 1 s after image acquisition started. Each measurement lasted 5 s with a minimal of 30 s interval in between consecutive measurements. The imaging system had a spatial resolution of 46 μm pixel$^{-1}$ and approximately 120 pixels were subtended by an average pupil.

Custom image processing (Fan et al. 2009) was applied to automatically calculate the pupil size from each image frame recorded during the 5 s acquisition. This method used a histogram-based thresholding (Fan et al. 2009) to segment pupils from eye images. The accuracy of pupil size calculation was on the scale of the pixel resolution of the imaging system (46 μm). To characterize pupillary response, the following six PLR parameters were calculated: (1) the resting pupil radius ($R_0$); (2) the minimal pupil radius ($R_m$) during constriction; (3) the percentage constriction calculated as $\Delta A = (R_0^2 - R_m^2)/R_0^2$; (4) the latency ($T_L$) calculated as the time interval between stimulus onset and the beginning of pupil constriction; (5) the constriction time ($T_C$) calculated as the time interval between the beginning of pupil constriction and when pupil reached minimal diameter $R_m$; (6) the redilation time ($T_R$) calculated as the time interval between the minimal diameter $R_m$ and when the pupil recovered to half of the constriction.

The PLR recording procedure in the MA and CP tests is illustrated in figure 1. As described before, each test was divided into three different test phases: pre-test, test, and post-test.

2.3. Heart rate and blood pressure monitors

Each participant’s HR was measured by a HR monitor (RS800CX, Polar, Kempele, Finland) at 1 kHz acquisition rate during the entire test session. Participant’s blood pressure was also measured periodically throughout the test via a blood pressure monitor (VSM 6000 series, Welch Allyn, Skaneateles Falls, NY, USA). Two blood pressures were taken in each segment of the test, for a total of 12 readings per subject. Blood pressures were taken from the left arm. Subjects were asked to minimize movement throughout the test to improve blood pressure acquisition.

All measurements obtained during each of the pre-test, test, and post-test phases were averaged to calculate the mean arterial blood pressure (MAP) and HR of each participant during each phase.

2.4. Procedure

2.4.1. Mental arithmetic. Before starting the MA task (‘pre-test’ phase), subjects were first asked to read aloud numbers shown on the computer screen for 3 min in order to establish an attention baseline. The numbers were integer numbers from 5–8700. At the 3 min mark, eight measurements were taken while the subjects continued to read the numbers.

Participants were then given instructions to solve either math problems or visual patterns that appeared on the screen. The problems consisted of simple addition, subtraction,
multiplication, division, and algebra. The visual pattern questions depicted four segments of a pattern and asked the participant to identify the fifth segment. Participants were informed that their answers would be recorded. They were given a maximum of 8 s to solve each problem. They were asked to say the solutions out loud and to solve the problems as quickly as possible. At the 3 min mark, eight measurements were taken while the subjects continued to solve problems.

Immediately after the problem solving section, subjects were asked to rate their feelings about the problems on a four level scale (1 = easy, 2 = ok, 3 = difficult, 4 = frustrated). Subjects were then shown scenic pictures selected to be easily visible through the red film. At the 3 min mark, eight measurements were taken while the subjects continued to look at the pictures. Subjects rested for 10 min between the mental stress and CP sections of the test.

2.4.2. Cold pressor. For the CP portion of the test, four baseline measurements were taken while participants were shown scenic pictures on the screen. Participants were then asked to place their right hand and forearm in cold water (5 °C) for 2 min. Four more measurements were taken during submersion. At the end of 2 min, the subjects were instructed to remove their hand and dry it. Immediately after the test, participants were asked to subjectively rate their pain level during the test on a four degree scale from 1 = none to 4 = severe. Subjects were allowed to rest for 2 min. At the end of the rest period, four measurements were taken.

2.5. Data processing and statistical analysis

The Kolmogorov–Smirnov test (Massey 1951) was used to verify normal distributions of all measured parameters. A repeated measures ANOVA model (PROC ANOVA procedure in SAS) was used to test the effects of test phase, gender, as well as their interaction. Test phase was treated as the within-subject effect and gender was treated as a between-subject effect. Post hoc paired t-test was used to confirm effects of tasks and one way ANOVA model was used to confirm gender effect. A p value <0.05 was considered significant.

3. Results

The total score for state anxiety ranged from 20–46 (30.9 ± 6.4), and scores for trait anxiety ranged from 20–52 (34.4 ± 8.3). Neither male subjects (state = 31.1 ± 7.9, trait = 32.3 ± 8.4) nor female subjects (state = 30.6 ± 4.9, trait = 36.8 ± 7.4) differed significantly from the sample of college students reported by Spielberger et al (1970). There was no significant difference between male and female subjects on state or trait anxiety (student t-test t9 = −0.17, p = 0.9 and t19 = 1.27, p = 0.2 for state anxiety and trait anxiety, respectively). The Kolmogorov–Smirnov test indicated that all measured PLR parameters, MAP and HR values followed a normal distribution at every test condition.

3.1. Mental arithmetic task

Subjects reported an average score of (3.4 ± 0.9) on the subjective difficulty scale administered at the conclusion of the MA section. Females reported a significantly higher difficulty rating (4.0 ± 0.0) than male subjects (2.8 ± 0.9) (Student t-test t9 = −4.12, p < 0.01).

3.1.1. Blood pressure and heart rate. The repeated measures ANOVA reported significant test phase effect (F2, 28 = 6.19 p = 0.006) and significant gender effect (F1, 14 = 4.85 p = 0.045) on MAP, while the interaction between gender and test phase was not significant. Males showed
higher mean baseline MAP than females (figure 2(a)). During the MA task, MAP increased significantly in both genders. In females, the MAP decreased during the recovery phase to a level lower than that of the pre-test (paired $t$-test between recovery and pre-test: $t_7 = 3.25$, $p = 0.014$) (figure 2(a)). A similar trend was observed in males, but did not reach statistical significance.

The repeated measures ANOVA also reported significant test phase effect on minimal pupil radius $R_m$ ($F_{2, 36} = 3.38$, $p = 0.045$), constriction $C_A$ ($F_{2, 36} = 6.35$, $p = 0.005$), latency $T_L$, (e) constriction time $T_C$, and (f) recovery time $T_R$. Error bars indicate standard error.

### 3.1.2. PLR parameters

During the pre-test phase, females and males had similar PLR parameters (figure 3). A trend for higher PLR constriction $C_A$ in males than females was observed (figure 3(c)), though the difference was not statistically significant (Student $t$-test $t_{18} = -1.30$, $p = 0.21$).

The repeated measures ANOVA reported significant test phase effect on minimal pupil radius $R_m$ ($F_{2, 36} = 3.38$, $p = 0.045$), constriction $C_A$ ($F_{2, 36} = 6.35$, $p = 0.005$), latency $T_L$, (e) constriction time $T_C$, and (f) recovery time $T_R$. Error bars indicate standard error.
Figure 4. The effects of cold pressor task on (a) mean arterial blood pressure (MAP) and (b) heart rate (HR). Error bars shown indicate standard error.

$T_L (F_{2,36} = 3.39, p = 0.045)$, constriction time $T_C (F_{2,36} = 5.79, p = 0.007)$ and recovery time $T_R (F_{2,36} = 8.35, p = 0.001)$. Neither gender nor the interaction between gender and test phase had a significant effect on any PLR parameters.

The average recovery time $T_R$ and constriction time $T_C$ decreased during the MA task in both males and females (figure 3). However, these decreasing trends reached statistically significance only in males on $T_R$ (paired $t$-test $t_9 = 3.85, p = 0.004$). The resting pupil size $R_0$, minimal pupil radius $R_m$, and constriction $C_A$ had an increasing trend during the MA task in females only, but these increases did not reach statistical significance in paired $t$-test.

During the recovery phase, the recovery time $T_R$ returned to the pre-test value in males. However $T_R$ did not change in females and was still significantly smaller than the pre-test value (paired $t$-test between pre-test and recovery: $t_9 = 3.47, p = 0.007$). $T_C$ continued to decrease into the recovery phase in females (paired $t$-test between pre-test and recovery: $t_9 = 2.97, p = 0.016$), but not in males. $C_A$ increased in both gender groups during the recovery period, but was significant only in females (paired $t$-test $t_9 = -3.54, p = 0.0063$ in females). The decreasing trend in $T_L$ continued into the recovery phase but only males had a significantly smaller $T_L$ than the pre-test phase (paired $t$-test $t_9 = 2.41, p = 0.039$ in males). During the recovery phase, $R_m$ remained the same in males but decreased in females and became smaller than the pre-test values (paired $t$-test between pre-test and recovery: $t_9 = 3.22, p = 0.011$).

3.2. Cold pressor task

Participants reported an average score of $(2.8 \pm 1.1)$ on the subjective pain scale administered after CP. There was no significant difference in subjective pain reported by males $(2.4 \pm 1.0)$ and females $(3.1 \pm 1.1)$.

3.2.1. Blood pressure and heart rate. The repeated measures ANOVA reported significant test phase effect ($F_{2,25} = 34.69, p < 0.0001$) and gender effect ($F_{1,13} = 7.29, p = 0.0182$) on MAP. The interaction between gender and test phase was not significant. The baseline MAP was higher in males than females (figure 4(a)). MAP increased significantly during CP in both genders (figure 4(a)) and dropped back to baseline during the recovery period.

The repeated measures ANOVA also revealed significant test phase effect ($F_{2,36} = 5.65, p = 0.007$) and gender effect ($F_{1,18} = 13.96, p = 0.002$) on average HR, while the interaction between gender and test phase was not significant. The baseline HR was higher in females than males (figure 4(b)). The HR increased during CP and recovered during the recovery period.
3.2.2. PLR parameters. The repeated measures ANOVA indicated that test phase had significant effects on base pupil radius $R_0$ ($F_{2, 36} = 20.12 p < 0.0001$) and minimal pupil radius $R_m$ ($F_{2, 36} = 19.49 p < 0.0001$). The interaction between gender and test phase was significant for PLR latency $T_L$ ($F_{2, 36} = 4.13 p = 0.032$) and constriction time $T_C$ ($F_{2, 36} = 3.61 p = 0.049$). No other effects or interactions were found significant.

The base pupil radius $R_0$ and minimal pupil radius $R_m$ increased in both genders (figures 5(a) and (b) respectively) during the CP submersion. However this increase was significant only in males for $R_0$ (paired t-test $t_9 = -6.86, p < 0.0001$) while the increase in $R_m$ was significant for both genders (paired t-test $t_9 = -5.48, p = 0.0004$ in males and $t_9 = -2.50, p = 0.034$ in females). In males, the constriction time $T_C$ increased during CP (paired t-test $t_9 = -3.72, p = 0.005$) but showed a decreasing trend in females which was not significant (figure 5(e)). PLR latency $T_L$ did not change significantly during CP submersion in either gender (figure 5(d)). The PLR constriction $C_A$ decreased slightly in females during CP submersion, but this change was not statistically significant (figure 5(c)). The redilation time $T_R$ showed an increasing trend in males but a decreasing trend in females although neither reached statistical significance in paired t-test.

During the recovery phase, $R_0$, $R_m$, and $T_C$ all recovered to the pre-test level in both genders. $T_R$ in males reversed the increase trend in CP and recovered back to the pre-test level; whereas it continued to decrease in females although its value was still not significantly different from the pre-test value. The latency $T_L$ decreased during recovery period in females and became smaller with respect to pre-test (paired t-test $t_9 = 2.38, p = 0.041$).

4. Discussion

Consistent with previous reports (Hellstrom and Lundberg 2000), females had a lower resting blood pressure which suggested a lower sympathetic activity and was attributed to greater baroreflex inhibitive control of sympathetic activity in females (Hogarth et al 2007). In addition, females had a higher resting HR due to vagal withdrawal (Hogarth et al 2007).
The MAP and HR increased in both genders during both the MA and CP tasks. This observation has been frequently documented in literature (Sloan et al 1991, Willemsen et al 2000, Tanida et al 2004, Freeman 2006, Zygmunt and Stanczyk 2010, Liu et al 2011). These changes suggest an elevated sympathetic activation during these two tasks. Notably, the MAP increased more significantly in CP than MA (26.4% versus 3.3% increase in females and 18.9% versus 4.5% increase in males during CP task), suggesting that CP has a stronger effect on sympathetic activation than MA.

The resting pupil size is controlled by the balance between the sympathetic tone and parasympathetic tone. The sympathetic activation during MA and CP shifts the original balance and increases the resting pupil size as reported in previous studies (Tavernor et al 2000, Yamanaka and Kawakami 2009). The increase in pupil size is related to the strength of stimulus or task demand (Hess and Polt 1964, Beatty 1982, Steinhauer et al 2004). In this study, the resting pupil size only showed an increasing trend in females during MA, most likely because females considered MA more challenging in this study. In CP, both genders reported a similar pain level. However males had much larger increases in resting pupil size than females during CP (5.8% increase in males versus 2.3% in females). This seems to be consistent with the prior observation that males show more sympathetic activity than females under stress (Dart et al 2002, Sato and Miyake 2004).

Besides the resting pupil size, other PLR parameters provide assessment of the dynamic ANS activation induced by the flash light stimulation. The constriction time and redilation time showed noticeable changes during the two tasks. Females had decreased constriction time in both MA and CP. Because the constriction amplitude was relatively stable, a faster pupil constriction process indicated that MA and CP induced stress accelerated parasympathetic activation in females. This seems to be in agreement with previous reports of increased parasympathetic response in females under stress (Sato and Miyake 2004, Nugent et al 2011). However, it is interesting to note that in males the constriction time increased in CP. As shown in previous studies, stress may induce more sympathetic increase in males (Dart et al 2002, Sato and Miyake 2004). Therefore, an elevated sympathetic activity may have suppressed the parasympathetic activation in males during CP and slowed the pupillary constriction.

Under the traditional model of autonomic pupillary control (Barbur 2004), pupil constriction is under the control of the parasympathetic nervous system, and pupil dilation is under the control of the sympathetic nervous system. Due to the stress-induced imbalance in the parasympathetic and sympathetic system, one would think a different trend would have been observed in the constriction and redilation times. However, the same trend (decrease or increase) was observed in both constriction time and redilation time across both test conditions and genders. An explanation for this correlation may lie in the timeline of constriction and dilation; parasympathetic withdrawal is believed to influence the first stage of redilation (Bremner 2009). In other words, the redilation time measured in this study may still be influenced by the parasympathetic activity.

5. Conclusion

Both MA and CP tests were able to elicit changes in HR, MAP, as well as in PLR parameters. Our results showed that static and dynamic parameters of the pupil are modulated differently by the changes in autonomic nervous system. The resting pupil size is more strongly affected by the sympathetic nervous system. In addition, males and females have differing levels of sympathetic and parasympathetic tone, which predisposes them to react to the same stimulus in different ways. Males generally have a higher sympathetic tone than females at rest, and they appear more likely to produce a sympathetic nervous response to a stressor. Females generally
have a higher parasympathetic tone than males at rest, and thus are more likely to mount a parasympathetic response to stressful stimuli. These results provide useful information to further validate and understand the effects of autonomic nervous system modulation on the pupil light reflex.

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