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14. ABSTRACT
We evaluated 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. 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. Both ASD and TD groups showed robust PLR-related activation in primary visual sensory areas including lateral geniculate nucleus [$F(1,40) = 16.3$, $p < .0005$] and striate cortex [$F(1,40) = 17.8$, $p < .0005$]. We found significant group differences in PLR-related activation in the cerebellum as well as anterior insula and superior frontal gyrus [$F(1,40) > 20$, $p < .00005$ in all instances].

15. SUBJECT TERMS
Pupillary light reflex, autism, functional MRI

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Table of Contents

1. INTRODUCTION

This project was designed 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 project. In Task #1, we propose to test 200 human subjects including 100 children with autism ("ASD" group), 65 typically developing children ("TD" group), 35 children with early brain dysfunction unrelated to autism ("MR" group). 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.

2. KEYWORDS

Autism spectrum disorder; Pupillary light reflex; Heart rate variability; Functional MRI; Autonomic nervous system; cerebellum; Neurodevelopmental disorder.

3. OVERALL PROJECT SUMMARY

3.1. 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.

The PLR test protocol has been described in Daluwatte et al. (2012a). 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 pupillogram (change in pupil size with time shown in Fig.1). The following PLR parameters were measured: the initial pupil diameter D_0, the maximal constriction diameter D_m, the PLR latency t_L (interval between stimulus onset and beginning of constriction), the constriction time t_C (interval between constriction 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 $(D_0^2-D_m^2)/D_0^2$.

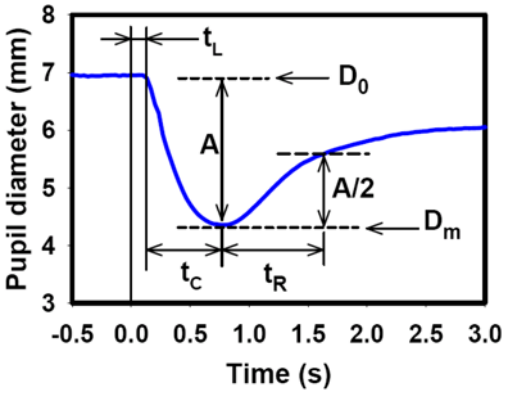

Fig.1. An illustration of the pupillogram and extracted PLR parameters. Please see text for explanations of each parameter.

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. 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.

The major results have been recently published in Journal of Autism and Developmental Disorders (Daluwatte et al. 2013). 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). Other age-related trends were described in detail in Daluwatte et al. (2012b).

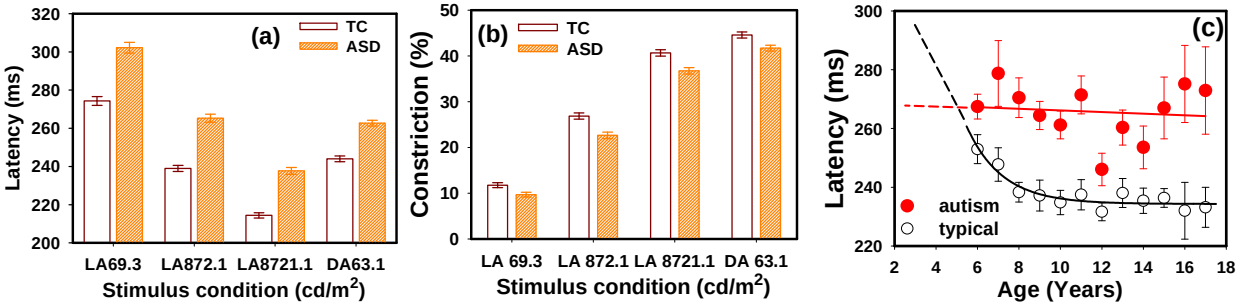

Fig. 2. Longer PLR latency (a) and lesser PLR constriction (b) in children with ASD than children of typical development obtained at various stimulation intensities. (c) PLR latency vs. age measured in the TC and ASD groups at light-adapted condition with stimulus intensity of LA872.1 cd/m^2. The lines 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 (Daluwatte 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.

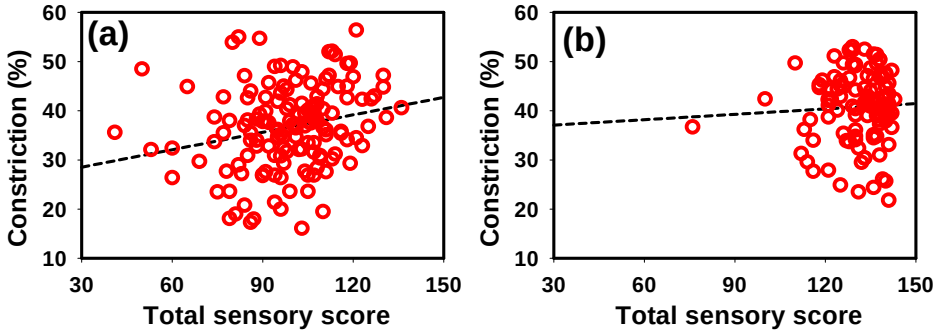

Fig. 3. The correlation between PLR constriction amplitude (at LA 8721.1 cd/m^2 stimulus intensity) and total sensory score in the (a) ASD and (b) TD groups. The Spearman rank correlation r = 0.26, p <0.01 in the ASD group; r = 0.003, p >0.05 in the TD group at LA 8721.1 cd/m^2. Lower sensory scores 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 adress 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).

3.2. 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.

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. In our protocol, 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.

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 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. 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.

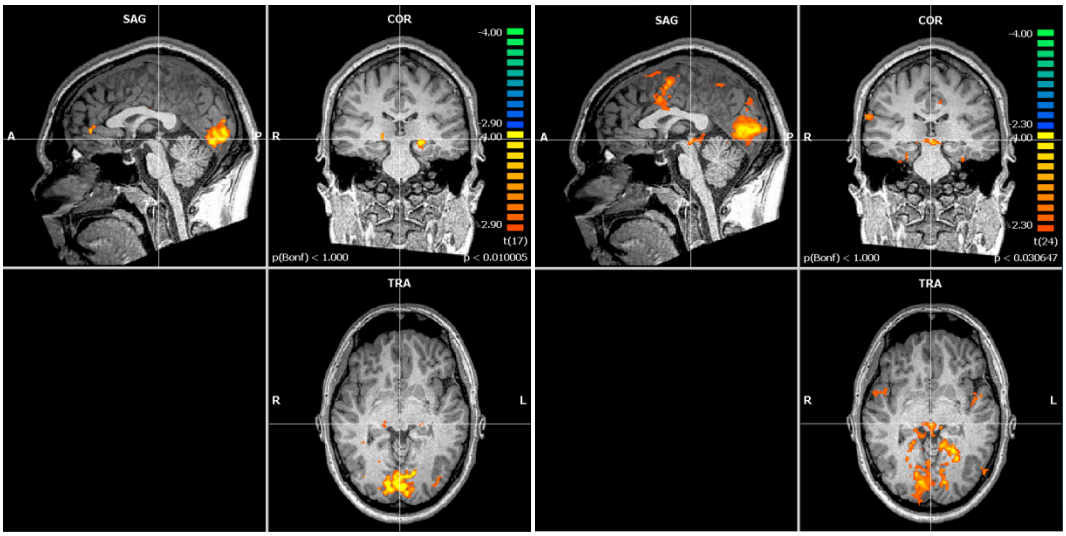

Fig. 4. PLR-related activation shown separately for the non-ASD group (left panel) and ASD group (right panel). Note the activation in the cortical visual areas and also the lateral geniculate nucleus of the thalamus.

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, 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. We then re-analyzed the fMRI data using a novel, more time-intensive approach that allowed 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.

After correcting motion artifact as described above, we saw robust PLR-related activation in both groups in primary visual sensory areas including lateral geniculate nucleus [$F(1,40) = 16.3$, $p < .0005$] and striate cortex [$F(1,40) = 17.8$, $p < .0005$]. PLR-related activation was also observed in association areas including superior parietal cortex [$F(1,40) = 17.4$, $p < .0005$] and right lateral prefrontal cortex [$F(1,40) = 9.0$, $p < .05$]. Most importantly, group differences in PLR-related activation were evident in the cerebellum as well as anterior insula and superior frontal gyrus, [$F(1,40) > 20$, $p < .00005$ in all instances]. The detailed results have been reported at 2014 International Meeting for Autism Research (Christ et al. 2014).

We have also undertaken a secondary analysis comparing cerebellum structure between the ASD group and control group. The part of cerebellum was manually segmented from all structure MRI images. A Matlab program was developed to further analyze the cerebellum

images. First the cerebellum volume was calculated by counting all image pixels with the cerebellum. The white matter was segmented using a histogram-based segmentation as described by Liu et al. (2003). The total volume of white matter was then calculated by counting all image pixels within the segmented white matter. In addition, the skeletal structure of the white matter was constructed by applying a thinning algorithm on the segmented white matter. The fractal dimension of the white matter skeletal was calculated using a classical box-counting algorithm.

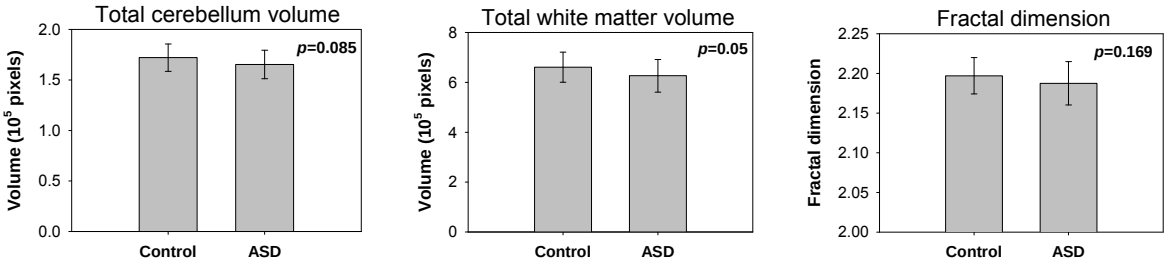

Fig. 5. Comparison of structural properties of cerebellum between the ASD and control groups.

As shown in Figure 5, the total cerebellum size was marginally larger in the control group (p=0.085), and the total white matter volume was larger in the control group (p=0.05). However, we did not see any difference in the fractal dimension between the two groups.

4. 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 found different activation in multiple brain regions in association with pupillary light reflex in children with ASD.

5. CONCLUSION

Our 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 and significant group differences in the cerebellum as well as anterior insula and superior frontal gyrus.

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.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

- **Peer-Reviewed Scientific Journals**

1. B.C. Davis, C. Daluwatte, N.C. Colona, G. Yao, "Effects of cold-pressor and mental arithmetic on pupillary light reflex," Physiol. Meas. 34:873-882 (2013). doi:10.1088/0967-3334/34/8/873
2. C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, N. Takahashi, G. Yao, "Atypical pupillary light reflex and heart rate variability in children with autism spectrum disorder," J. Autism and Developmental Disorders 43:1910-1925 (2013). doi:10.1007/s10803-012-1741-3
3. C. Daluwatte, J. H. Miles, G. Yao, "Simultaneously measured pupillary light reflex and heart rate variability in healthy children," Physiol. Meas. 33:1043-1048 (2012). doi:10.1088/0967-3334/33/6/1043

- **Proceeding Papers**

4. C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, A. Lofgreen, N. Birliner, G. Yao, "Age-dependent pupillary light reflex in children with autism," Conf Proc IEEE Eng Med Biol Soc. 2012:3776-9 (2012). doi:10.1109/EMBC.2012.6346789

- **Conference Abstracts**

5. S. E. Christ, A. Moffitt, C. Daluwatte, M. Price, J.H. Miles, & G. Yao, "The neural basis for atypical pupillary light response in autism spectrum disorder," 2014 International Meeting for Autism Research, May 14 – May 17, Atlanta, GA.
6. J. H. Miles, N. Takahashi, C. Daluwatte, G. Yao, "Pupillary Light Reflex Parameter Constriction Amplitude Relationship to Clinical Symptoms," 2013 International Meeting For Autism Research, 2 - 4 May, Donostia/San Sebastián, Basque Country, Spain.
7. C. Daluwatte, J. H. Miles, J.G. Sun, G. Yao, "Association between Sensory Behavior and Pupillary Light Reflex in Children with Autism Spectrum Disorders," 2013 International Meeting For Autism Research, 2 - 4 May, Donostia/San Sebastián, Basque Country, Spain.
8. C. Daluwatte, J. H. Miles, J.G. Sun, G. Yao, "Association between pupillary light reflex and sensory behavior in children with autism spectrum disorders," 2013 ACRT/SCTS Joint Annual Meeting, April 17-19,2013, Washington, DC.
9. (*)C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, N. Takahashi, G. Yao, "Atypical pupillary light reflex and heart rate variability in children with autism," 2012 International Meeting For Autism Research, Toronto, Ontario, May 17-19, 2012.
10. (*)C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, N. Takahashi, G. Yao, "Simultaneous measurement of pupillary light reflex and heart rate variability in children with autism," 2011 International Meeting For Autism Research, Philadelphia, PA, May 19-22, 2011.

7. INVENTIONS, PATENTS AND LICENSES

- G. Yao, J. H. Miles, D. M.R. Dinalankara, "Device to Measure Pupillary Light Reflex In Infants and Toddlers," US patent application number US20140063461 A1, filing date Nov. 8, 2013.

8. REPORTABLE OUTCOMES

- Pupillary light reflex (PLR) device: this study establishes that PLR tests can be used for screening neurodevelopmental disorders.

9. OTHER ACHIEVEMENTS

- Degree obtained: Chathuri Daluwatte, Ph.D., May, 2013.
- New funding: Yao (PI) with J.H. Miles , "Evaluation of pupillary light reflex as a biomarker for neurodevelopmental disorders in young children," National Institute of Child Health & Human Development, 1R21HD075971, 07/08/2013 – 04/30/2015,

10. REFERENCES

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Christ, S.E., A. Moffitt, C. Daluwatte, M. Price, J.H. Miles, & G. Yao, (2014). The neural basis for atypical pupillary light response in autism spectrum disorder. 2014 International Meeting for Autism Research, May 14 – May 17, Atlanta, GA.

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Davis, B.C., Daluwatte, C., Colona, N.C., Yao, G. (2013). Effects of cold-pressor and mental arithmetic on pupillary light reflex. Physiol. Meas. 34, 873-882.

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11. APPENDICES

- **Journal publications**

1. B.C. Davis, C. Daluwatte, N.C. Colona, G. Yao, "Effects of cold-pressor and mental arithmetic on pupillary light reflex," Physiol. Meas. 34:873-882 (2013).
2. C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, N. Takahashi, G. Yao, "Atypical pupillary light reflex and heart rate variability in children with autism spectrum disorder," J. Autism and Developmental Disorders 43:1910-1925 (2013).
3. C. Daluwatte, J. H. Miles, G. Yao, "Simultaneously measured pupillary light reflex and heart rate variability in healthy children," Physiol. Meas. 33:1043-1048 (2012).

- **Conference papers**

4. C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, A. Lofgreen, N. Birliner, G. Yao, "Age-dependent pupillary light reflex in children with autism," Conf Proc IEEE Eng Med Biol Soc. 2012:3776-9 (2012).

- **Conference presentations**

5. S. E. Christ, A. Moffitt, C. Daluwatte, M. Price, J.H. Miles, & G. Yao, "The neural basis for atypical pupillary light response in autism spectrum disorder," 2014 International Meeting for Autism Research, May 14 – May 17, Atlanta, GA.
6. S.E. Christ, R.M. Zamzow, G. Yao, J.D. Johnson, "Resting state functional network organization and topological properties in autism spectrum disorder," 2013 International Meeting For Autism Research, 2 - 4 May, Donostia/San Sebastián, Basque Country, Spain.
7. C. Daluwatte, J. H. Miles, J.G. Sun, G. Yao, "Association between Sensory Behavior and Pupillary Light Reflex in Children with Autism Spectrum Disorders," 2013 International Meeting For Autism Research, 2 - 4 May, Donostia/San Sebastián, Basque Country, Spain.
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ORIGINAL PAPER

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

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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

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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

significantly 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 when stimulated using a constant light intensity. This observation was confirmed by Fan et al. (2009a) using a short 100 ms optical stimulus at several different intensities. 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,

Table 1 Distribution of IQ and medication use in TD, ASD and NDD groups

Group	IQ	w/o med	w/med
TD	High-IQ	98	0
	Low-IQ	2	0
ASD	High-IQ	44	34
	Low-IQ	23	21
ASD diagnosis			
Asperger	High-IQ	9	11
	Low-IQ	2	0
Autism	High-IQ	23	14
	Low-IQ	19	15
PDD-NOS	High-IQ	12	9
	Low-IQ	2	6
NDD	High-IQ	9	9
	Low-IQ	7	9

High-IQ: IQ score of 80 or higher (at or above 9.1th percentile)
Low-IQ: IQ score lower than 80 (below 9.1th percentile)

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/m^2, LA 872.1 cd/m^2, and LA 8721.1 cd/m^2. The dark-adapted (DA) PLR was then measured at a stimulation intensity of DA 63.1 cd/m^2 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 (D_0), defined as the average resting pupil diameter before stimulus onset; (2) the relative constriction amplitude, calculated as $A_{\%} = \left(D_0^2 - D_m^2\right)/D_0^2$, where D_m is the minimal pupil diameter during constriction; (3) the latency (t_L), defined as the time that elapsed between stimulus onset and the beginning of pupil constriction; (4) the constriction time (t_C), defined as the time interval between the beginning of pupil constriction and when pupil reached minimal diameter D_m; (5) the redilation time (t_R), calculated as the time interval between the minimal diameter D_m and when the pupil recovered to half of the constriction; (6) the constriction velocity (v_C), calculated as $(D_0 - D_m)/2t_C$; and (7) the redilation velocity (v_R), calculated as $(D_0 - D_m)/4t_R$. 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). Two

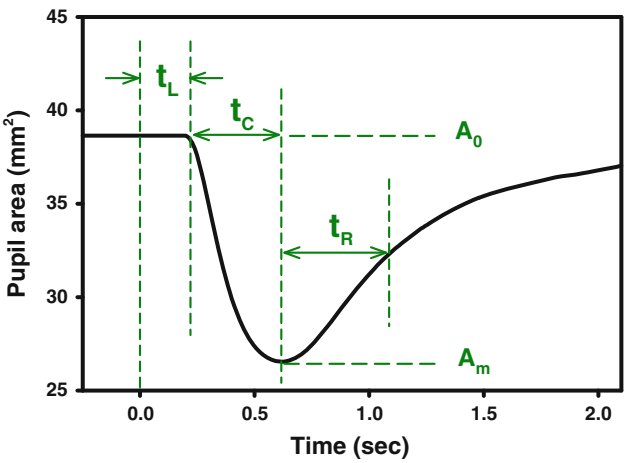

Fig. 1 An illustration of the pupilogram and the associated PLR parameters. The optical stimulus is given at time zero. The baseline and minimal pupil diameters are calculated as $D_0 = 2\sqrt{A_0/\pi}$ and $D_m = 2\sqrt{A_m/\pi}$, respectively. The relative constriction amplitude is obtained as $A_{\%} = (A_0 - A_m)/A_0$. The constriction and redilation velocities are calculated as $v_C = (D_0 - D_m)/2t_C$ and $v_R = (D_0 - D_m)/4t_R$, respectively

frequency-domain HRV parameters were calculated: the normalized power of the high-frequency band (HF_N) (HF = 0.15–0.4 Hz) and the LF/HF power ratio, where the low-frequency bandwidth was 0.04–0.15 Hz. HF_N 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 5 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 ($\hat{\eta}^2_{\Lambda,r=1}$) for group differences using MANOVA. An $\hat{\eta}^2_{\Lambda,r=1}$ 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 (t_C), relative constriction amplitude ($\Delta A_{\%}$), latency (t_L), redilation time (t_R), 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 ($F_{2,1107} = 150.44$ $p < 0.0001$), relative constriction amplitude ($F_{2,1106} = 29.96$ $p < 0.0001$), constriction time ($F_{2,1103} = 31.69$ $p < 0.0001$), and redilation time ($F_{2,1096} = 14.67$ $p < 0.0001$). Post-hoc MANOVA confirmed that the ASD and NDD groups had a significantly longer latency ($F_{4,229} = 23.24$ $p < 0.0001$ $\hat{\eta}^2_{\Lambda,r=1} = 0.28$ for ASD; $F_{4,130} = 21.69$ $p < 0.0001$ $\hat{\eta}^2_{\Lambda,r=1} = 0.38$ for NDD) and lesser relative constriction amplitude ($F_{4,231} = 4.47$ $p = 0.002$ $\hat{\eta}^2_{\Lambda,r=1} = 0.06$ for ASD; $F_{4,130} = 3.74$ $p = 0.007$ $\hat{\eta}^2_{\Lambda,r=1} = 0.08$ for NDD) than those of the TD group for all testing conditions. The ASD group also had a shorter constriction time (MANOVA $F_{4,228} = 5.01$ $p = 0.0007$ $\hat{\eta}^2_{\Lambda,r=1} = 0.06$) and redilation time (MANOVA $F_{4,225} = 3.39$ $p = 0.01$ $\hat{\eta}^2_{\Lambda,r=1} = 0.04$) 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 $F_{4,152} = 1.71$ $p = 0.15$). 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 ($F_{2,1343} = 50.81$ $p < 0.0001$) and on time-domain

Table 2 Summary of PLR results

Stimulus intensity (cd/m^2)	Resting pupil diameter (mm)	PLR latency (ms)*	Constriction (%)*	Constriction time (ms)*	Redilation time (ms)*	Constriction velocity (mm^2/s)	Redilation velocity (mm^2/s)
TD							
LA 69.3	6.58 ± 0.61	274.3 ± 23.9	11.8 ± 5.5	370.7 ± 73.4	402.0 ± 86.3	0.81 ± 0.44	0.37 ± 0.20
LA 872.1		239.0 ± 16.2	26.9 ± 7.1	399.2 ± 52.8	498.1 ± 99.9	1.75 ± 0.75	0.72 ± 0.30
LA 8721.1		214.4 ± 14.4	40.8 ± 7.2	464.5 ± 51.2	595.6 ± 116.6	2.37 ± 0.95	0.98 ± 0.45
DA 63.1	7.44 ± 0.77	244.0 ± 15.4	44.7 ± 6.9	580.2 ± 59.4	804.4 ± 171.8	2.41 ± 0.97	0.91 ± 0.36
ASD							
LA 69.3	6.50 ± 0.81	302.2 ± 32.2	9.6 ± 6.1	336.7 ± 75.6	370.3 ± 89.2	0.76 ± 0.50	0.35 ± 0.21
LA 872.1		265.3 ± 25.4	22.5 ± 8.3	364.5 ± 63.4	455.8 ± 104.0	1.65 ± 0.76	0.68 ± 0.31
LA 8721.1		237.7 ± 22.5	36.6 ± 8.5	434.1 ± 62.9	560.7 ± 106.1	2.39 ± 0.91	0.95 ± 0.35
DA 63.1	7.47 ± 0.88	262.7 ± 18.5	41.6 ± 7.7	552.1 ± 73.8	737.8 ± 155.0	2.55 ± 0.91	0.99 ± 0.40
NDD							
LA 69.3	6.36 ± 0.74	307.1 ± 44.6	10.5 ± 5.8	372.9 ± 77.1	415.7 ± 117.4	0.65 ± 0.46	0.29 ± 0.19
LA 872.1		270.9 ± 22.1	22.1 ± 7.3	379.4 ± 60.1	475.5 ± 86.8	1.42 ± 0.86	0.55 ± 0.34
LA 8721.1		245.6 ± 23.6	36.5 ± 8.8	446.0 ± 66.8	562.2 ± 85.6	1.99 ± 1.01	0.78 ± 0.34
DA 63.1	7.14 ± 0.83	269.9 ± 26.2	42.2 ± 8.5	566.3 ± 107.4	730.0 ± 116.5	2.00 ± 0.85	0.79 ± 0.35

The results are represented as group mean ± standard deviation

* Significant group difference (ANCOVA $p < 0.0001$)

Table 3 Summary of HRV results

HRV measurement phase	AHR (bmp)*	SDNN (ms)*	rMSSD (ms)*	LF/HF (n.u.)	HF_N (%)
TD					
1	90.1 ± 12.4	64.2 ± 24.3	38.2 ± 20.5	2.7 ± 1.6	31.2 ± 10.3
2	90.0 ± 12.1	69.7 ± 24.3	36.9 ± 17.7	4.5 ± 5.2	22.8 ± 8.0
3	93.1 ± 12.5	66.4 ± 28.0	32.6 ± 17.1	3.4 ± 1.6	26.3 ± 10.0
4	91.0 ± 13.0	72.0 ± 26.3	36.4 ± 18.2	4.4 ± 3.0	22.7 ± 9.5
5	92.9 ± 13.3	65.4 ± 27.2	33.2 ± 17.7	4.1 ± 0.3	25.2 ± 10.5
ASD					
1	95.2 ± 14.0	56.9 ± 20.1	32.2 ± 15.6	2.9 ± 1.8	29.5 ± 10.8
2	96.1 ± 13.7	61.1 ± 20.9	31.4 ± 15.6	3.7 ± 2.1	24.7 ± 9.9
3	99.8 ± 12.9	56.8 ± 23.4	27.4 ± 14.0	3.4 ± 1.7	25.2 ± 8.3
4	97.0 ± 13.0	60.6 ± 23.8	30.6 ± 15.5	3.7 ± 1.9	24.1 ± 8.4
5	99.4 ± 13.4	57.1 ± 23.3	28.0 ± 13.9	3.8 ± 2.6	24.3 ± 9.3
NDD					
1	100.1 ± 13.8	51.4 ± 18.3	27.9 ± 12.6	2.4 ± 1.2	32.6 ± 10.4
2	98.9 ± 13.1	54.6 ± 18.2	28.6 ± 12.3	3.1 ± 1.6	27.3 ± 9.1
3	105.3 ± 13.4	44.8 ± 13.6	22.1 ± 9.1	3.0 ± 1.3	26.9 ± 7.5
4	101.5 ± 13.8	51.5 ± 19.2	26.7 ± 12.2	3.7 ± 2.0	24.6 ± 9.6
5	104.7 ± 14.5	45.9 ± 15.4	23.3 ± 12.3	3.5 ± 1.8	25.5 ± 9.4

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

bmp 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 ($F_{2,1340} = 41.92$ $p < 0.0001$; and $F_{2,1340} = 27.46$ $p < 0.0001$ 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 ($F_{5,218} = 3.32$ $p = 0.007$ $\hat{\eta}^2_{\Lambda,r=1} = 0.05$) (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 $F_{5,146} = 2.63$ $p = 0.03$ $\hat{\eta}^2_{\Lambda,r=1} = 0.05$). The NDD group also had a significantly faster AHR ($F_{5,132} = 5.41$ $p = 0.0001$ $\hat{\eta}^2_{\Lambda,r=1} = 0.14$), lower SDNN ($F_{5,131} = 4.70$ $p = 0.0006$ $\hat{\eta}^2_{\Lambda,r=1} = 0.12$) and lower rMSSD ($F_{5,131} = 2.63$ $p = 0.03$ $\hat{\eta}^2_{\Lambda,r=1} = 0.06$) 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/m^2, LA 872.1 cd/m^2, LA 8721.1 cd/m^2, and DA 63.1 cd/m^2, respectively). However, this decreasing trend did not exist in the ASD group at any of the 4 stimulus intensities ($F_{1,36} = 0.37$ $p = 0.55$; $F_{1,35} = 0.07$ $p = 0.79$; $F_{1,37} = 0.08$ $p = 0.93$; and $F_{1,35} = 0.06$ $p = 0.81$).

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 R^2 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 HF_N 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. HF_N 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

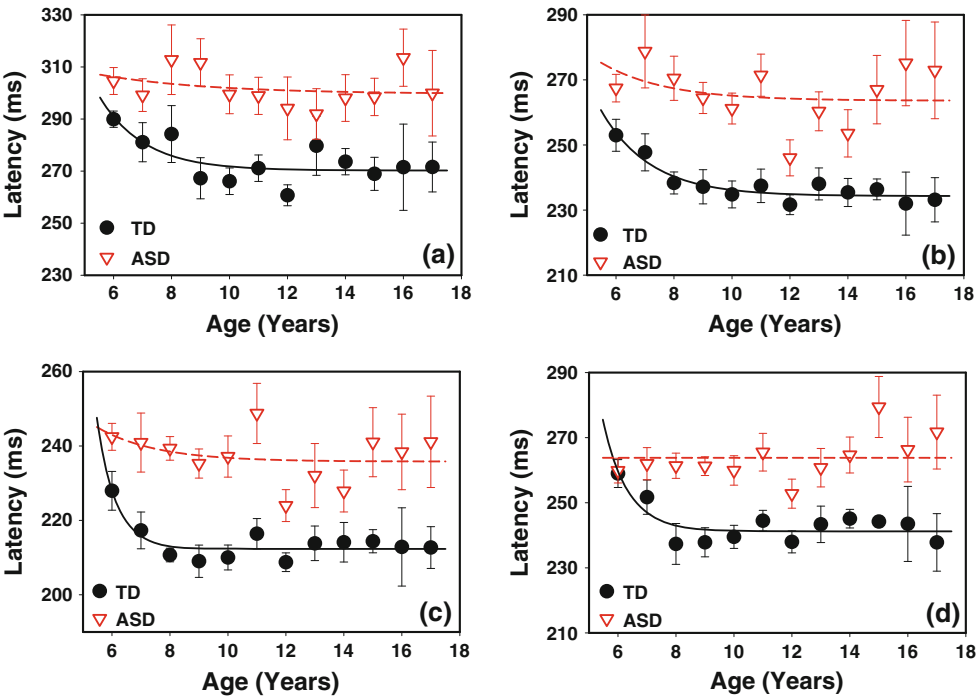

Fig. 2 PLR latency versus age measured in the TD and ASD groups at different stimulus conditions: **a** LA 69.3 cd/m^2, **b** LA 872.1 cd/m^2, **c** LA 8721.1 cd/m^2, **d** DA 63.1 cd/m^2. The *lines* are fitting results using an exponential decay function y = a*exp(−b*x) + c. The *error bars* indicate the standard error

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 ($F_{5,156} = 2.23$ $p = 0.006$) 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.

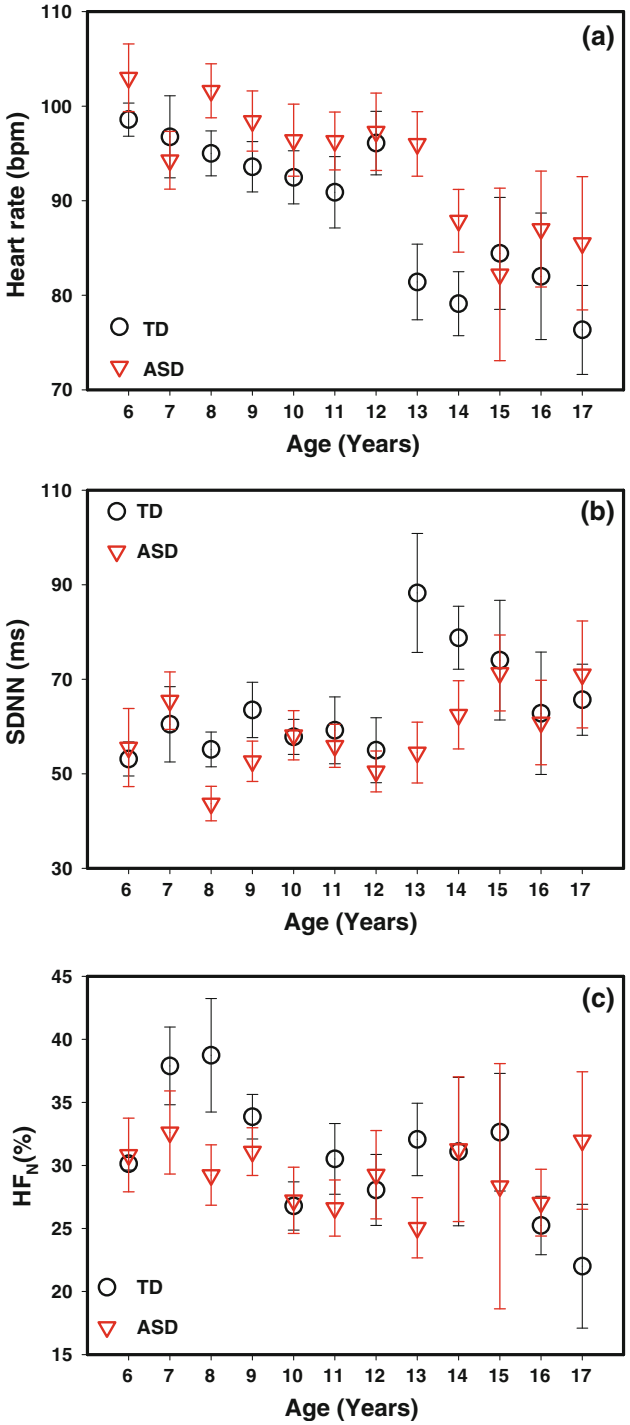

Fig. 3 The **a** average heart rate, **b** SDNN and **c** HF_N obtained at different ages in the TD and ASD groups measured during the HRV measurement phase 1 (before PLR test). Similar results were obtained in other HRV measurement phases. The *error bars* indicate the standard error

Children with ASD and a "Low IQ" had a slower mean AHR, larger SDNN and rMSSD than those with a "High

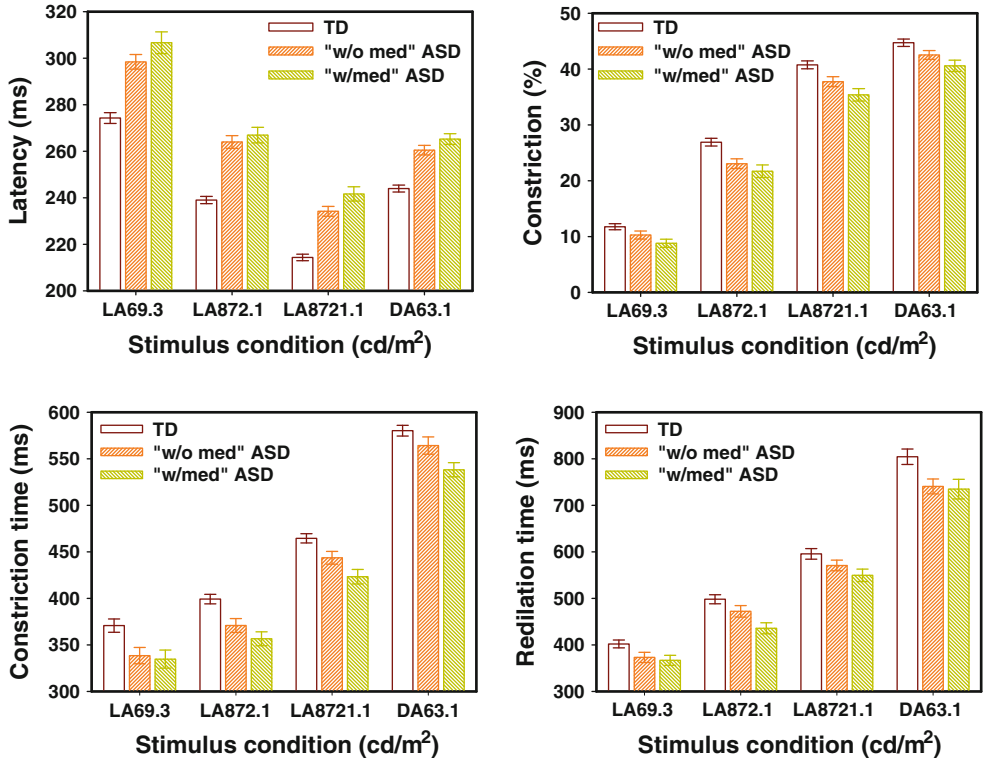

Fig. 4 PLR latency, constriction amplitude, constriction time and redilation time in the TD, "w/med" ASD, and "w/o med" ASD groups measured at different stimulus conditions. The *error bars* indicate the standard error

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 transiting 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 ($F_{4,1343} = 6.29$ $p < 0.0001$; $F_{4,1340} = 3.29$ $p = 0.01$; $F_{4,1340} = 4.89$ $p = 0.0006$; $F_{4,1340} = 8.84$ $p < 0.0001$; 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

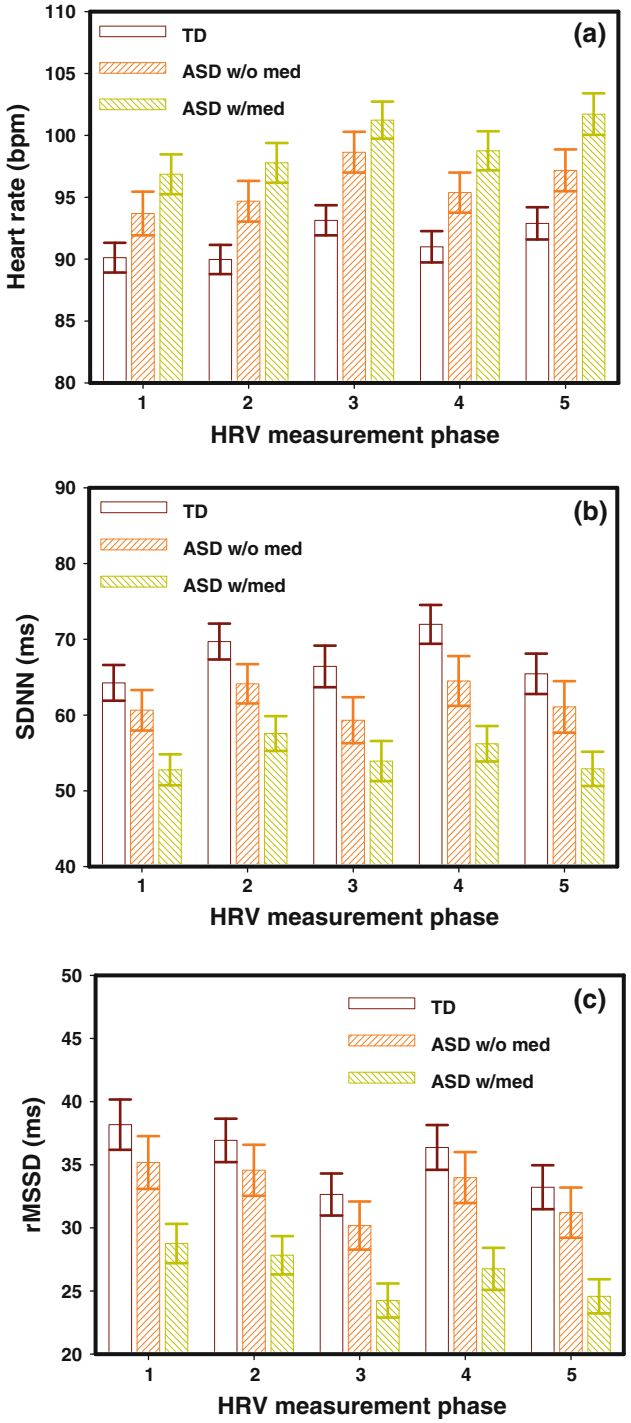

Fig. 5 The **a** average heart rate, **b** SDNN and **c** rMSSD in the TD, "w/med" ASD and "w/o med" ASD groups obtained in the five HRV measurement phases. The HRV measurement phases are numbered as *1* before PLR test, *2* during LA PLR, *3* during dark adaptation, *4* during DA PLR, and *5* after PLR test. The *error bars* indicate the standard error

Fig. 9. HF_N 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_N. The HF_N 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_N 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/m^2. 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/m^2 and LA 8721.1 cd/m^2, constriction amplitude at LA 69.3 cd/m^2 and LA 8721.1 cd/m^2, constriction time at LA 69.3 cd/m^2 and LA 872.1 cd/m^2, 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.

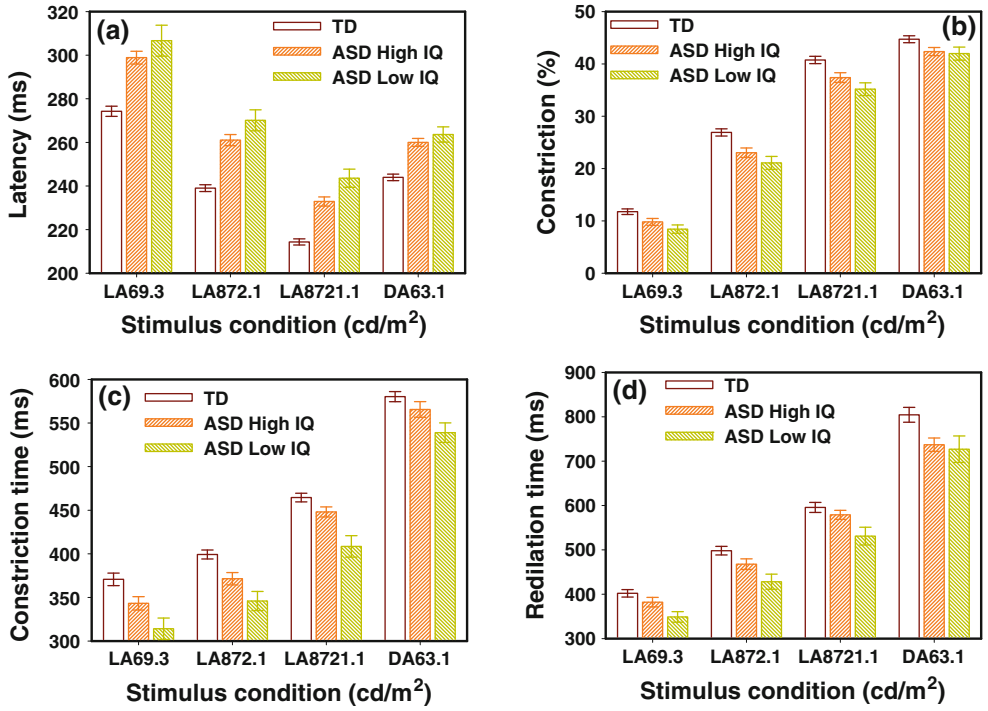

Fig. 6 The IQ effects on **a** PLR latency, **b** constriction amplitude, **c** constriction time and **d** redilation time in the ASD group. The *error bars* indicate the standard error

Discussion

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).

We also observed a significant age effect on HRV parameters which has been widely reported previously (Massin and von Bernuth 1997; Silvetti et al. 2001). The average heart rate is known to decrease with age, and time-domain HRVs (SDNN and rMSSD) were reported to increase with age (Silvetti et al. 2001). Massin and von Bernuth (1997) showed that HRV parameters changed rapidly during the first few years of life and eventually stabilized at older ages (6–15 years). Such age effects on HRV were generally attributed to the progressive maturation of the ANS (Silvetti et al. 2001). Our results suggested that the age effect on HRV was similar in the TD and ASD groups (Fig. 3), which is in sharp contrast to the different age profiles observed in 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

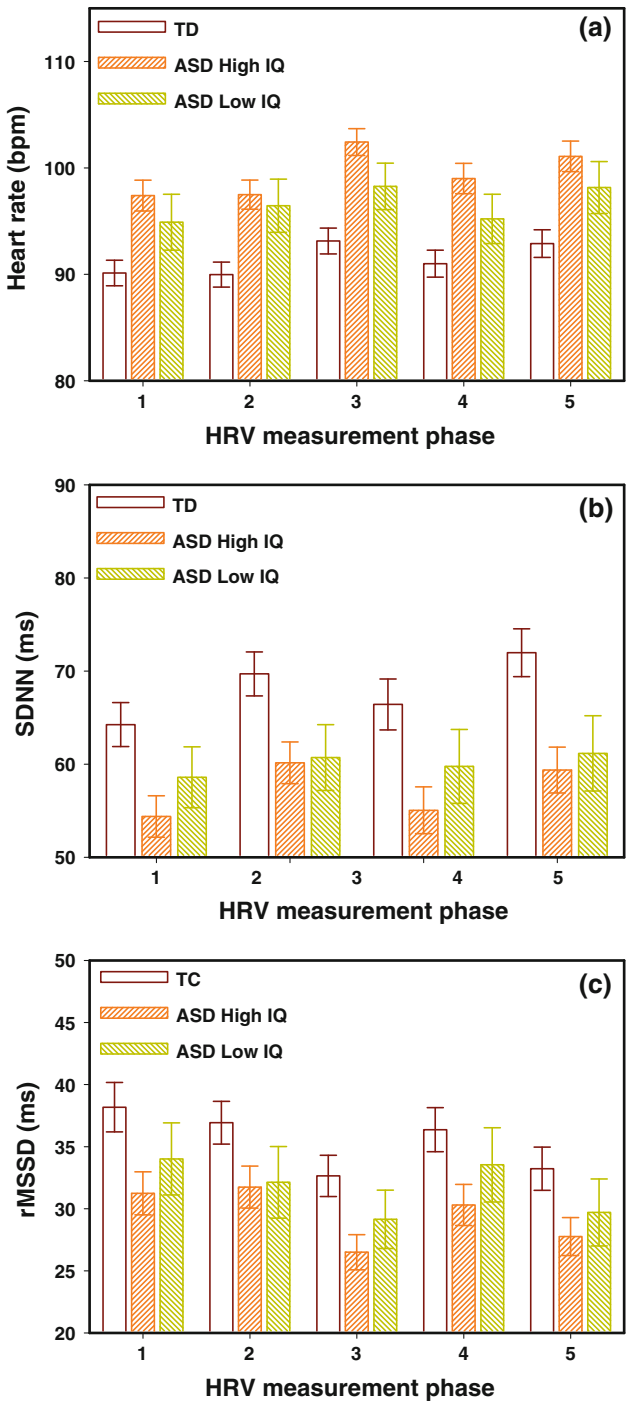

Fig. 7 The IQ effects on **a** AHR, **b** SDNN and **c** rMSSD in the ASD group obtained in all five HRV measurement phases. The HRV measurement phases are numbered as *1* before PLR test, *2* during LA PLR, *3* during dark adaptation, *4* during DA PLR, and *5* after PLR test. The *error bars* indicate 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 HF_N 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 ($\sim 15°$), 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

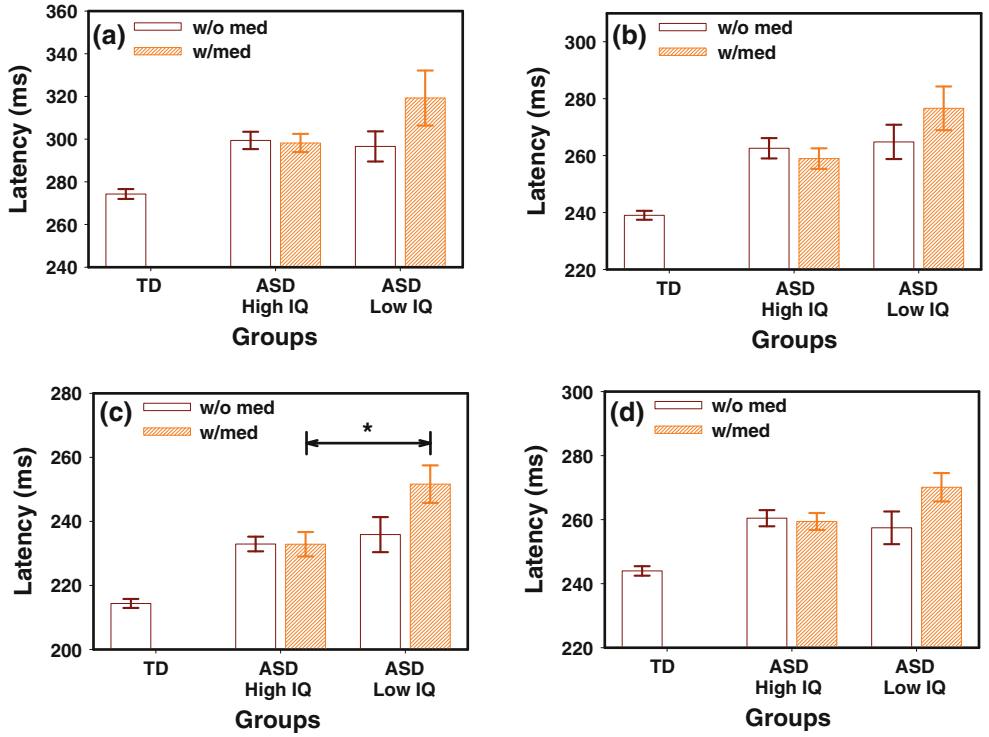

Fig. 8 The effect of IQ and medication interaction on PLR latency at stimulation intensities of **a** LA69.3 cd/m^2, **b** LA872.1 cd/m^2 **c** LA8721.1 cd/m^2, and **d** DA63.1 cd/m^2 in the ASD group. *t test $p = 0.03$, Bonferroni corrected

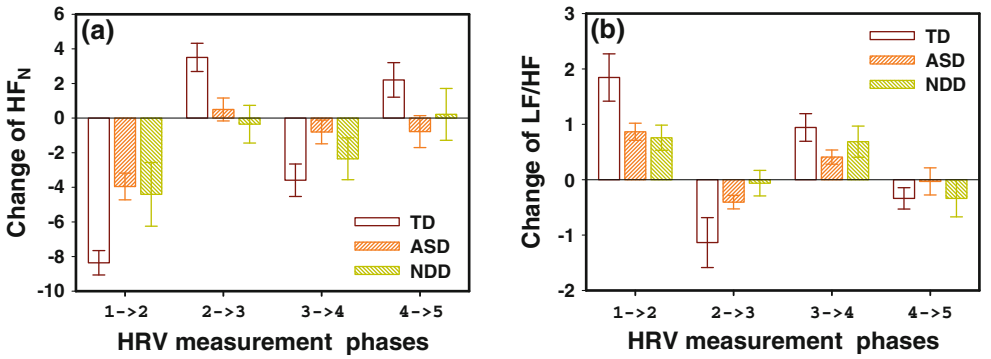

Fig. 9 The change of frequency domain HRV parameters between consecutive HRV measurement phases. **a** HF normalized power and **b** LF/HF ratio. The *error bars* indicate the standard error. The HRV measurement phases are numbered as *1* before PLR test, *2* during LA PLR, *3* during dark adaptation, *4* during DA PLR and *5* after PLR test

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.

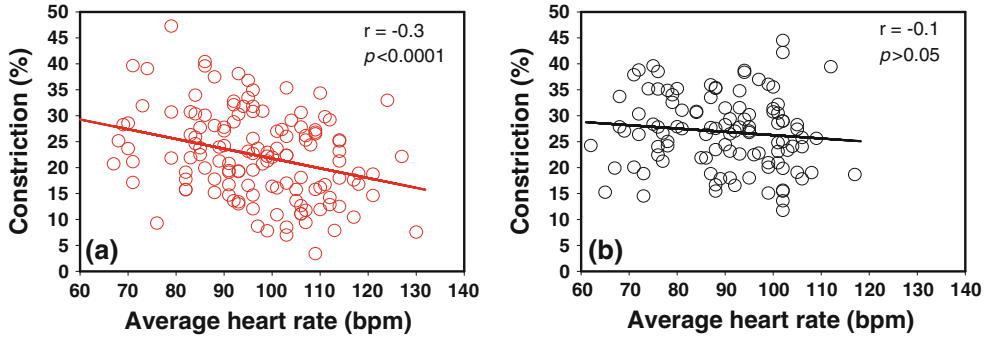

Fig. 10 The correlation between average heart rate and relative constricition amplitude in **a** children with ASD and **b** typical controls. The data shown were measured at stimulus intensity of LA 872.1 cd/m^2. (Pearson's r = −0.3*, −0.3**, −0.3**, −0.1 [a]in the ASD group and r = −0.06[a], −0.1[a], −0.1[a], −0.02[a] in the TD group at stimulus LA 69.3 cd/m^2, LA 872.1 cd/m^2, LA 8721.1 cd/m^2, and DA 63.1 cd/m^2, respectively. **$p < 0.001$, *$p < 0.01$, [a]$p > 0.05$)

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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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 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 increases 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 $\pm$ 0.8 years old) and ten females (19.9 $\pm$ 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 3.3×10^{-5} W m^{-2} and a field size of 5.3° 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 light stimulus 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

Stressor	Mental arithmetic						Rest	Cold pressor			
Test phase	Pre-test		Test		Post-test			Pre-test	Test	Post-test	
Duration (min)	3	5	3	5	3	5	10	2	2	2	2
PLR test		YES		YES		YES		YES	YES		YES

Figure 1. An illustration of the PLR measurements during the mental arithmetic and cold pressor tests. Each test was divided into three phases: pre-test, test, and post-test.

(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 ($T_{\mathrm{L}}$) 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 $\pm$ 6.4), and scores for trait anxiety ranged from 20–52 (34.4 $\pm$ 8.3). Neither male subjects (state = 31.1 $\pm$ 7.9, trait = 32.3 $\pm$ 8.4) nor female subjects (state = 30.6 $\pm$ 4.9, trait = 36.8 $\pm$ 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 $t_{18} = -0.17$ $p = 0.9$ and $t_{18} = 1.27$, $p = 0.2$ 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 $\pm$ 0.9) on the subjective difficulty scale administered at the conclusion of the MA section. Females reported a significantly higher difficulty rating (4.0 $\pm$ 0.0) than male subjects (2.8 $\pm$ 0.9) (Student *t*-test $t_9 = -4.12$, $p < 0.01$).

3.1.1. Blood pressure and heart rate. The repeated measures ANOVA reported significant test phase effect ($F_{2,\,28} = 6.19$ $p = 0.006$) and significant gender effect ($F_{1,\,14} = 4.85$ $p = 0.045$) on MAP, while the interaction between gender and test phase was not significant. Males showed

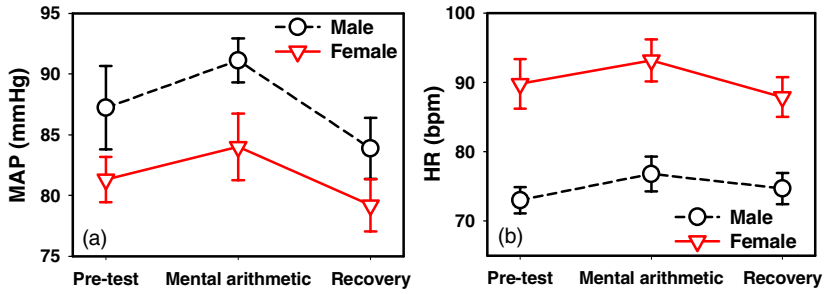

Figure 2. The effects of mental arithmetic on (a) mean arterial blood pressure (MAP) and (b) heart rate (HR). Error bars shown indicate standard error.

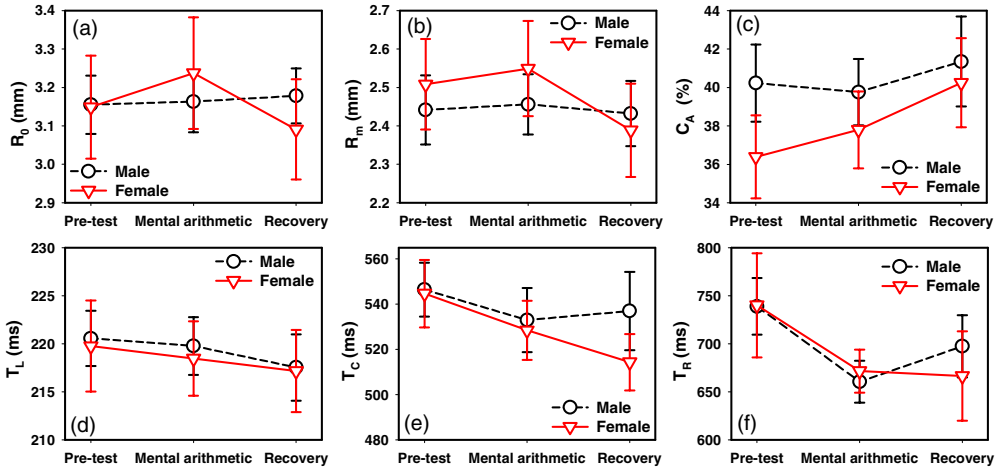

Figure 3. The effects of mental arithmetic task on PLR parameters including (a) resting pupil radius R_0, (b) minimal pupil radius R_m, (c) relative constriction C_A, (d) latency T_L, (e) constriction time T_C, and (f) recovery time T_R. Error bars indicate standard error.

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 ($F_{2,\,36} = 8.58$ $p = 0.0009$) and gender effect ($F_{1,\,18} = 17.49$ $p = 0.0006$) on average HR, while the interaction between gender and test phase was not significant. Males had lower HR than females (figure 2(b)). During the MA task, HR increased significantly and during the recovery phase, recovered to pre-test level in both genders.

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

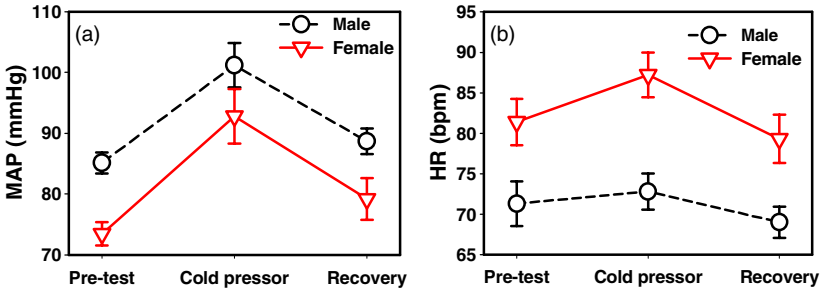

Figure 4. The effects of cold pressor task on (a) mean arterial blood pressure (MAP) and (b) heart rate (HR). Error bars shown indicate standard error.

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 ± 1.1) on the subjective pain scale administered after CP. There was no significant difference in subjective pain reported by males (2.4 ± 1.0) and females (3.1 ± 1.1).

3.2.1. Blood pressure and heart rate. The repeated measures ANOVA reported significant test phase effect ($F_{2,26} = 34.69$ $p < 0.0001$) 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.

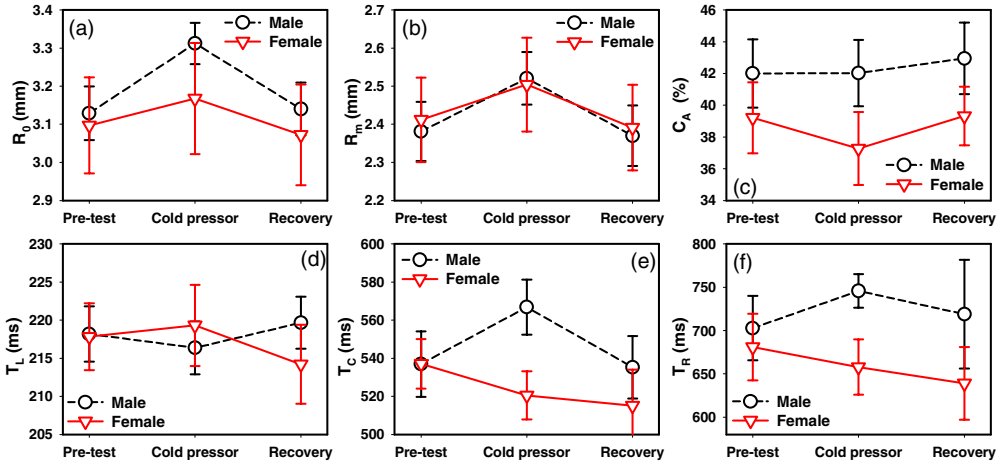

Figure 5. The effects of cold pressor task on PLR parameters including (a) resting pupil radius R_0, (b) minimal pupil radius R_m, (c) relative constriction C_A, (d) latency T_L, (e) constriction time T_C, and (f) recovery time T_R. Error bars indicate standard error.

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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Simultaneously measured pupillary light reflex and heart rate variability in healthy children

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Abstract

We investigated the potential inter-relationship between two measures of autonomic nervous system: pupillary light reflex (PLR) and heart rate variability (HRV), in healthy children of 8–16 years old. PLR was measured at both dark- and light-adapted conditions with various stimulation intensities. Simultaneously measured HRV was obtained in five different PLR testing phases: before PLR test, light-adapted PLR test, dark adaptation, dark-adapted PLR test and after PLR test. The frequency domain HRV parameters measured during the PLR test were significantly different from those measured during rest. Both the regression analysis and factor analysis indicated that PLR and HRV parameters were not correlated, which suggests that they may provide complementary assessment of different aspects of the overall autonomic nervous system.

Keywords: pupillary light reflex, heart rate variability, children

(Some figures may appear in colour only in the online journal)

1. Introduction

The autonomic nervous system (ANS) is a complex and pervasive system that controls the critical visceral functions of the human body and is involved in many psychophysiological responses. Its two divisions, the sympathetic and parasympathetic systems, act in a complementary manner regulated by the central autonomic network (Levy 1997). In such a highly integrated organization, ANS dysfunctions are often widespread with symptoms appearing in multiple subsystems. Hence, an interesting question arises: whether an assessment of a specific ANS subsystem may reflect the overall physiological status of the system, and how variations in different measures of ANS correlate with each other (Bär *et al* 2009). This study investigated the possible inter-relationship between two specific ANS measures: pupillary light reflex (PLR) and heart rate variability (HRV).

PLR refers to the change of pupil size in response to luminance changes. The pupil size is controlled by two iris muscles, the sphincter and dilator, which produce pupil constriction and dilation, respectively. The sphincter is mainly innervated by the parasympathetic nervous system (PNS), whereas the dilator is innervated by the sympathetic nervous system (SNS) (Barbur 2004). The parasympathetic nerve fibers synapse in the ciliary ganglion and control the sphincter muscle via the short ciliary nerve (Lowenstein and Loewenfeld 1950). These fibers originate from the pupilloconstrictor neurons at the Edinger–Westphal (EW) nucleus that receives input from the olivary pretectal nucleus (OPN) in the midbrain. The dilator muscles are controlled by post-ganglionic sympathetic fibers from superior cervical ganglion that receives input from the ciliospinal center of Budge (Appenzeller 1999). The pupil size is determined by the balance between the sympathetic and parasympathetic systems (Fotiou *et al* 2000). Abnormal PLR has been observed in many neurological disorders associated with ANS dysfunction (Bremner 2009) such as panic disorder (Kojima *et al* 2004), autism (Fan *et al* 2009b) and Parkinson's disease (Giza *et al* 2011, Stergiou *et al* 2009).

HRV assesses the beat-to-beat variations of the heart rate. Both parasympathetic and sympathetic systems are involved in cardiovascular system regulation. Stimulation of the parasympathetic fibers (vagus nerves) reduces the heart rate, whereas the sympathetic stimulation increases the heart rate through the sinoatrial (SA) node. HRV parameters have been widely applied to evaluate cardiac autonomic functions (Kamath and Fallen 1993) and have been useful in identifying the ANS function in various allostatic systems (Thayer and Sternberg 2006). HRV has also been applied in evaluating the ANS dysfunction in disorders such as panic disorder (Yeragani *et al* 1993), schizophrenia (Bär *et al* 2005, Bär *et al* 2007) and sleep disorders (Bonnet and Arand 1998).

The possible association between PLR and HRV has recently been investigated by several authors. Correlations between HRV and PLR parameters were found in adults during exercise (Kaltsatou *et al* 2011) and in patients with acute schizophrenia (Bär *et al* 2008). However, Bär *et al* (2009) found limited correlation between specific PLR and HRV parameters in healthy adults 19–64 years old. It is unclear whether similar relationships may exist in younger subjects as both HRV and PLR are affected by age. Here we report a study that measured simultaneously the PLR and HRV in healthy 8–16 year-old children to further investigate the possible association between measures obtained from pupillary light reflex and heart rate variability (HRV).

2. Methods

2.1. Participants

A total of 54 healthy 8–16 year-old children participated in this study including 27 boys (138.2 $\pm$ 28.2 months) and 27 girls (age 136.1 $\pm$ 28.1 months) without any known vision, neurological and cardiovascular problems. All were tested at least 1 h after their last meal. Among the participants, 23 children (9 girls and 14 boys) were tested in the morning (8 am–12 pm) and the remaining 31 (18 girls and 13 boys) were tested in the afternoon (12 pm–5 pm). All participants and their legal guardians were thoroughly informed of the procedure and consented with a written informed consent as approved by the Institutional Review Board of the University of Missouri.

2.2. Instrument

A binocular pupilogram recording system was used to measure the PLR. This system is similar to that reported previously (Fan *et al* 2009a, 2009b) except that faster cameras were used to

Figure 1. An illustration of the test procedure used in this study.

record pupil images at 115 fps. The spatial resolution of the system was 35 μm/pixel. A 530 nm green LED provided the light stimulus. The stimulus pulse width was 100 ms. Neutral density filters and LED current were used to control the stimulation intensity.

To obtain HRV, real-time QRS intervals were recorded by using a remote heart rate measuring device (Polar RS800CX, Polar Electro Oy, Finland). A chest strap with enclosed heart rate sensor and wireless transmitter was wrapped around the participant's chest. The system acquires the ECG at 1 kHz rate. The heart beat QRS signals transmitted from the chest strap were received and recorded by a watch-like device. Multiple studies (Gamelin *et al* 2006, Gamelin *et al* 2008, Goodie *et al* 2000, Nunan *et al* 2009, Porto and Junqueira Jr 2009) have shown this device to be reliable for the short-term R–R interval measurement and to provide results consistent with traditional ECG.

2.3. Test procedure

Heart rate recording was started 5 min before the PLR test to acquire a baseline reference. The participant remained in a sitting position during the entire test. PLR was first measured in a light-adapted (LA) condition (30 cd m^{-2} room illumination). Three optical stimulation intensities were used in LA tests: 69.3, 872.1 and 8721.1 cd m^{-2} to induce different amounts of pupillary constriction. Following a 15 min dark adaptation (<0.02 cd m^{-2} room illumination), dark-adapted (DA) PLR was then measured at a stimulation intensity of 63.1 cd m^{-2}. At each test condition, the left eye was stimulated first and the right eye was stimulated next. Images of both pupils were recorded for the analysis. The measurements were repeated four times for each condition with a 20 s interval between measurements. Pupil imaging was started 1 s before the onset of the 100 ms optical stimulation to obtain baseline pupil size, and was recorded continuously for 4.5 s. Heart rate was continuously recorded during the PLR test and stopped 5 min after completing the PLR test. The entire test procedure is illustrated in figure 1.

2.4. Data analysis

The pupilogram was constructed by extracting the pupil size from acquired pupil images as described in detail previously (Fan *et al* 2009a, 2009b). As in our previous studies, the PLR parameters listed in table 1 were measured in this study to characterize PLR responses. PLR parameters obtained from both eyes during the four repeat measurements were averaged to calculate the mean value at any given condition.

HRV was assessed using both time domain and frequency domain methods (Malik *et al* 1996). As shown in table 2, two time domain parameters were calculated: SDNN and rMSSD. To be consistent with the most recent studies on HRV, two frequency domain parameters were analyzed: the normalized HF and the LF/HF ratio. The frequency domain power spectrum was analyzed by using fast Fourier transform (FFT) as described by Malik *et al* (1996). To study the potential effect of the PLR tests on HRV parameters, HRV was analyzed in five

Table 1. PLR parameters measured in this study.

Symbol (unit)	Definition
D_0 (mm)	'Base pupil diameter': pupil diameter before stimulus onset
$D_{\min}$ (mm)	'Minimal pupil diameter': pupil diameter before stimulus onset
$\Delta A_{\%}$	'Relative constriction amplitude': $\left(D_0^2 - D_{\min}^2\right)/D_0^2$
t_L (ms)	'Constriction latency': the elapsed time between light stimulus and beginning of constriction
t_C (ms)	'Constriction time': the time interval from the beginning of constriction to the maximal constriction
t_R (ms)	'Redilation time': the time interval between maximal constriction and recovery to half of the constriction $\left(D_0^2 - D_{\min}^2\right)/2$
v_C (mm s^{-1})	'Constriction velocity': average velocity of the relative constriction $(D_0 - D_{\min})/2t_C$
v_R (mm s^{-1})	'Recovery velocity': average velocity of the relative recovery $(D_0 - D_{\min})/(4t_R)$

Table 2. HRV parameters measured in this study.

Symbol (unit)	Definition
HR (bpm)	Average heart rate
SDNN (ms)	Standard deviation of normal RR intervals
rMSSD (ms)	Root mean square successive difference of the RR intervals
HF_n	Normalized powers of HF band (HF, 0.15–0.4 Hz), i.e. the relative powers of HF band when removing very low frequency (VLF, 0–0.04 Hz) band power from total power. HF power/(total power–VLF power) $\times$ 100%
LF/HF	The ratio of LF/HF power (LF, 0.04–0.15 Hz)

testing phases: before PLR test (5 min), LA test (10 min), dark adaptation (15 min), DA test (5 min) and after PLR test (5 min).

The distributions of PLR and HRV parameters were verified to conform to normal distributions using the Kolmogorov–Smirnov test. 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 test conditions, gender and age. *P*-values were Bonferroni-corrected appropriately using the number of stimulus conditions for PLR and the number of testing phases for HRV. To study the association between PLR and HRV parameters, linear correlations between them were first analyzed with Pearson product moment correlation (PROC CORR procedure in SAS). To investigate whether variations in PLR parameters can be explained by a combination of HRV parameters or vice versa, multilinear regression (PROC REG procedure in SAS) was applied with HRV (or PLR) parameters treated as dependent variables and PLR (or HRV) parameters treated as independent variables. Moreover, the explanatory factor analysis (PROC FACTOR procedure in SAS) was performed on the overall data set to study the potential relationships between PLR and HRV parameters using oblique matrix rotation. The factor analysis reduces a multivariate system to a system with a fewer number of dimensions by categorizing highly correlated dependent variables into a single factor.

3. Results

3.1. Pupillary light reflex

Table 3 shows the mean values and standard deviations of all measured PLR parameters. The initial pupil diameter D_0 increased from 6.6 $\pm$ 0.6 mm in LA to 7.5 $\pm$ 0.8 mm in DA. The

Table 3. Summary of PLR results. The results are represented as group mean ± standard deviation.

	Stimulation intensity (cd m^{-2})			
	LA 69.3	LA 872.1	LA 8721.1	DA 63.1
Resting pupil diameter (mm)		6.6 ± 0.6		7.5 ± 0.8
Relative constriction amplitude (%)	12.8 ± 6.0	27.0 ± 7.4	40.8 ± 7.89	44.6 ± 7.3
Latency (ms)	269.3 ± 26.6	236.1 ± 15.8	211.8 ± 13.5	239.3 ± 15.9
Constriction time (ms)	368.6 ± 71.8	398.5 ± 56.6	463.4 ± 52.8	575.6 ± 52.3
Redilation time (ms)	417.6 ± 74.7	501.7 ± 109.9	610.7 ± 135.8	817.8 ± 172.0
Constriction velocity (mm s^{-1})	1.0 ± 0.5	2.0 ± 0.7	2.8 ± 0.8	2.8 ± 0.8
Redilation velocity (mm s^{-1})	0.5 ± 0.2	0.8 ± 0.3	1.1 ± 0.3	1.0 ± 0.0

Table 4. Summary of HRV results. The results are represented as group mean ± standard deviation.

	Testing phase				
	Before PLR test	During LA PLR test	During DA period	During DA PLR test	After PLR test
Heart rate (bpm)	89.9 ± 12.1	90.1 ± 12.1	92.7 ± 12.1	91.0 ± 13.2	93.5 ± 13.2
SDNN (ms)	67.6 ± 27.0	70.2 ± 23.9	67.9 ± 29.1	71.5 ± 28.5	65.2 ± 25.0
rMSSD (ms)	38.9 ± 20.3	36.7 ± 16.9	34.1 ± 17.7	36.4 ± 18.6	33.2 ± 16.9
HF_n (%)	31.8 ± 11.0	23.1 ± 7.1	26.9 ± 10.1	22.3 ± 8.2	24.9 ± 10.1
LF/HF ratio	2.6 ± 1.4	3.8 ± 1.6	3.2 ± 1.4	4.1 ± 2.1	3.7 ± 2.3

relative constriction amplitude $\Delta A_{\%}$ increased from 12.8% ± 6.0% at LA 69.3 cd m^{-2} to 40.6% ± 7.3% at LA 8721.1 cd m^{-2}. The PLR latency was between 200 and 300 ms. In LA tests, PLR latency decreased with stimulus intensity (F-value = 236.0, $p < 0.0001$ in ANOVA test for a linear trend). $\Delta A_{\%}$ was similar at LA 8721.1 cd m^{-2} and DA 63.1 cd m^{-2}, but the DA latency was ∼30 ms longer. Average constriction velocity v_C ranged from 1.0 to 2.8 mm‘ s^{-1} at the four stimulation conditions, whereas the average redilation velocity v_R was 0.5–1.0 mm s^{-1}.

3.2. Heart rate variability

The HRV parameters are shown in table 4. The average heart rate was around ∼ 90 bpm in our study population. The time domain HRV parameter SDNN was ∼65–72 ms on average and the rMSSD ranged between 33 and 39 ms. The frequency domain HRV parameters were HF normalized power (∼22–32) and LF/HF ratio (∼2–4).

3.3. Inter-relationship between PLR and HRV

As shown in table 4, the average heart rate, SDNN and rMSSD measured during different PLR testing phases were not significantly different (ANCOVA F-value = 1.06, 0.52 and 0.86, $p = 0.38$, 0.72 and 0.48, respectively). In fact, the HRV parameters obtained during different testing phases were highly correlated with each other. The heart rate had the highest correlation coefficient among all parameters. Table 5 shows that the correlation weakened as the measurement phase became distinct in time, i.e. the r-value decreased from 0.95 between phases 1 and 2 (5 min interval) to 0.88 between phases 1 and 5 (25 min interval).

However, the correlation of frequency domain HRV among different testing phases was not as high as the time domain parameters. The LF/HF ratio had the least correlation r-values (0.54–0.87) among different testing phases. In fact, the ANCOVA model indicated that the PLR testing phase had a significant fixed effect on the LF/HF ratio and HF normalized power

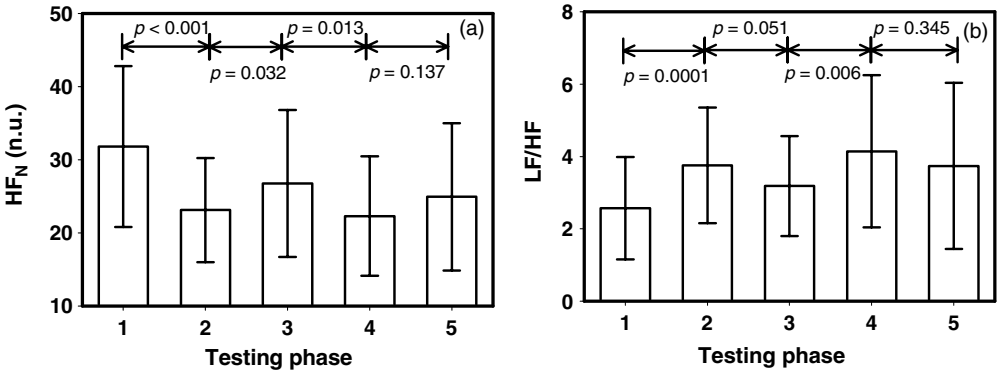

Figure 2. A comparison of (a) normalized HF power and (b) LF/HF ratio during different testing phases. *P*-values shown were obtained from the paired *t*-test. The testing phases are numbered as 1: before PLR test, 2: during LA PLR, 3: during dark adaptation, 4: during DA PLR, 5: after PLR test. The error bars indicate the standard deviation.

Table 5. Correlations between average heart rate measured in different testing phases. The results are represented as Pearson's *r*-value.

	Before test	LA test	DA	DA test	After test
Before test	1.00[a]	0.95[a]	0.91[a]	0.92[a]	0.88[a]
LA test		1.00[a]	0.94[a]	0.95[a]	0.90[a]
DA			1.00[a]	0.93[a]	0.91[a]
DA test				1.00[a]	0.90[a]
After test					1.00[a]

[a] $p < 0.0001$.

(ANCOVA F-value = 6.74 and 8.52, $p < 0.0001$ and $p < 0.0001$). As shown in figure 2, HF normalized power was significantly lower during the two PLR testing phases (both LA and DA tests) than during the three resting periods (before PLR test, dark adaptation and after PLR test), while the LF/HF ratio was significantly higher.

The Pearson product moment correlation did not show any significant correlation between simultaneously measured PLR parameters and HRV parameters ($p > 0.05$, $r < 0.5$ for all the correlations). Further multilinear regression analysis concluded that no significant model could be developed to explain variations in PLR parameters by using HRV parameters, nor vice versa.

Using the exploratory factor analysis with oblique matrix rotation (OBVARIMAX in SAS), the multivariate PLR and HRV dataset was reduced to a system of four factors which were uncorrelated with each other (inter-factor correlations $r < 0.3$). These four factors were determined such that the total variance of the dataset (proportion = 1) can be explained by the resulting system with a reduced dimension and also using the scree plot to identify the last substantial drop which is after factor 4 (Fabrigar *et al* 1999). The factor loading after oblique matrix rotation (OBVARIMAX in SAS) is shown in table 6, where each factor loading represents the correlation between a PLR or HRV parameter and the identified factor. Factors 1 and 2 were mainly attributed to loadings from HRV parameters. The heart rate and time domain HRV parameters (SDNN and rMSSD) had the highest correlation with factor 1, whereas the two frequency domain HRVs, LF/HF and HF_n, had the highest correlation with factor 2. PLR parameters were the major contributors to factors 3 and 4. Factor 3 had a high loading from t_C and t_R, while factor 4 had moderately high correlations with t_L and D_o. The reliability of each variable was evaluated by analyzing 'Cronbach's coefficient of alpha when deleted' with

Table 6. Factors loading for the entire data set by using the factor analysis. The numbers represent correlation between PLR/HRV parameters and the identified factors.

		Factor 1	Factor 2	Factor 3	Factor 4
	RMSSD	−0.93	−0.19	−0.03	0.02
	SDNN	0.95	−0.07	0.00	−0.02
HRV	HR	0.81	0.02	0.06	−0.02
	LF/HF ratio	−0.02	−0.90	0.10	0.01
	HF_n	0.01	0.94	0.03	0.01
	Constriction time	−0.01	0.00	0.93	−0.07
PLR	Redialtion time	0.02	−0.05	0.91	0.09
	Latency	0.02	−0.03	0.04	0.42
	Initial pupil diameter	−0.02	0.02	−0.02	0.34

'PROC CORR'. The results indicated that the heart rate should be removed from factor 1. In addition, a 'Cronbach's coefficient of alpha' of 0.7 was used as the acceptable threshold to evaluate the reliability of the entire factor system. The results indicated that the final solution should only consist of factors 1 and 3, where factor 1 represented the HRV system and factor 3 represented the PLR system. Communalities of selected parameters over the final factor solution were above 0.8 for all the parameters selected in the factor solution. Such consistently high communalities suggested that the sample size of the design had a minimal effect on the factor solution (MacCallum *et al* 1999). The numbers shown in table 6 were calculated by using PLR data obtained at LA 8721.1 cd m^{-2} and HRV data obtained during the LA testing phase. However, the same conclusion was reached for all other testing conditions.

4. Discussion

The PLR parameters obtained in this study are similar to those reported previously (Fan *et al* 2009b) in a similar age group at similar stimulus conditions. Similarly, the time domain HRV parameters obtained in this study are consistent with those reported in previous studies (Umetani *et al* 1998). Our mean HF_n (%) is smaller than that reported by Gamelin *et al* (2008) in boys younger than 11 years in supine position. We think that these differences can be attributed to the seated position used in our study. It is known that HRV measured at supine position has higher HF_n (%) and smaller LF/HF than that measured at standing position (Montano *et al* 1994). The HRV parameters obtained in seated position are in between of those obtained at supine and standing positions (Chan *et al* 2007).

Because 43% of participants were tested in the morning and the rest were tested in the afternoon, we examined the potential effect of different time of day of the test. The results showed no significant difference between the PLR and HRV measurements obtained in children tested in the morning and those tested in the afternoon (t-test, $p > 0.15$). The HRV results (table 4) measured in phases 2 and 3 (figure 1) had longer measurement time (10 min and 15 min, respectively) than the other three phases (5 min each). To investigate whether such a difference may lead to different HRV values, we divided the 10 min LA test period into two 5 min segments and calculated HRV parameters in each segment. The obtained HRV parameters in the two 5 min segments were similar to each other (paired t-test, $p > 0.28$) and were also similar to the one calculated over the entire 10 min period. The same results were obtained for the HRV parameters obtained in the 15 min DA period.

The frequency domain HRV parameters were clearly affected by the PLR testing phases. The mean results (table 4) indicate that HF_n decreased 27.3% and LF/HF increased 45.9%

when going from 'before test' to PLR test. This trend is similar to posture-induced HRV changes that are related to sympathetic activity caused by orthostatic stress (Mukai and Hayano 1995, Montano *et al* 1994, Yeragani *et al* 1993). Because the participant slightly inclined forward (∼15°) during the PLR tests, it is possible that such a posture change led to elevated sympathetic activity due to increased muscle stress. In addition, the PLR test itself may induce some task-related stress. Delaney and Brodie (2000) reported that psychological stress can decrease high-frequency HRV and increase low-frequency HRV. Vagal tone and respiratory sinus arrhythmia (RSA) are the principal contributors to the high-frequency power of HRV, while both vagal and sympathetic tones influence the low-frequency component (Berntson *et al* 1997). Overall the observed changes in frequency domain HRV parameters suggest lower vagal tone and increased sympathetic modulation during the PLR test.

PLR parameters and HRV parameters are categorized as independent and uncorrelated factors according to the factor analysis. The SDNN and RMSSD were closely aligned only with factor 1 and had very low loading on other factors (table 6). Similarly, the constriction time and redilation time were aligned only with factor 3. LF/HF and HF_n were highly aligned with factor 2 with high loading. However, with a smaller loading, PLR latency and initial pupil diameter were best aligned with factor 4. Therefore, the data clearly indicated a lack of association between PLR and HRV. This observation may be attributed to the fact that healthy typically developing children were tested in this study. In this group, many PLR parameters were quite consistent with very small between-subject variation. Notably, the coefficient of variation of PLR latency was $<10\%$, in agreement with the fact that latency did not contribute to the four-factor system derived from the common factor analysis.

The lack of association between PLR and HRV can be further corroborated by the different gender effect in PLR and HRV parameters. In agreement with a previous report by Krishnan *et al* (2009) in a large group of children of similar age range, our data showed that girls had a significantly higher heart rate than boys. A higher heart rate indicates stronger sympathetic influence (Malik *et al* 1996). We observed a trend of smaller resting pupil size in girls although the difference did not reach statistical significance. The same difference was reported by Fan *et al* (2009a) as statistically significant in individuals with a tightly controlled age range. A smaller resting pupil size may suggest a stronger parasympathetic modulation or a weaker sympathetic modulation (Barbur 2004). Summarizing the above comparisons, it is clear that gender effects in PLR and HRV do not completely corroborate with each other.

Although PLR and HRV are part of an integrated ANS, individual neurological pathways behave differently. The natural variations in an individual system may not affect other subsystems. However, this conclusion may not hold true for some disorders associated with ANS dysfunctions where pervasive ANS changes exist in multiple subsystems. For example, significant correlations between PLR and HRV were found in patients with acute schizophrenia (Bär *et al* 2008).

5. Conclusion

We measured PLR and HRV simultaneously in age- and gender-matched healthy children 8–16 years old. The gender effect was observed in both HRV and PLR parameters. However, the significant age effect was observed in HRV parameters but not in PLR. We found that the frequency domain HRV parameters were significantly different in different PLR testing phases, likely due to the psychological or/and physiological stress induced by the PLR tests. Using both the regression analysis and factor analysis, we conclude that variations of PLR and HRV are not associated in healthy children. However, this conclusion might be altered in the case of ANS disorders or other situations when pervasive changes might appear in multiple different

ANS subsystems. Nevertheless, PLR and HRV may provide a complementary assessment of different aspects of the overall autonomic nervous system.

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Age-dependent pupillary light reflex parameters in children*

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Abstract— **Pupillary light reflex (PLR) refers to the phenomenon where pupil size changes in response to stimulation with a flash of light. It is a simple functional test that can reveal dysfunctions associated with the PLR pathway. Although abnormal PLR responses have been reported in many neurological disorders, few studies investigated neurodevelopmental effects on PLR parameters. We studied the effect of age on PLR in a group of 6 to 17 year old children with typical development. A significant and consistent age effect was found on PLR latency in children younger than 10 years old. Age effects were also observed in resting pupil diameter and constriction amplitude. However such age related trends were not observed in children with neurodevelopment disorders. These results suggest that PLR has the potential to be used as a simple noninvasive tool for monitoring neurodevelopment in children.**

I. Introduction

American Academy of Pediatrics (AAP) estimates that 12% to 16% children have some forms of developmental disorders [1]. Substantial clinical evidence supports that early intervention leads to improved functioning. Early detection is essential to ensure early intervention [1]. In the United States developmental screening is presumed be done in the pediatrician's or family doctor's office using one or more screening questionnaires [2]. Unfortunately, this practice is neither consistent nor universal which leads to considerable lag in the diagnosis for children with developmental disabilities [1]. In addition, behavioral symptoms usually lag behind the underlying neurophysiological changes. Therefore there is a need for an objective measure that can accurately track normal neurodevelopment progress in children.

Pupillary light reflex (PLR) is tested by measuring pupil size change in response to a short light flash. The size of the pupil is controlled by two antagonistic iris muscles: the sphincter and the dilator that are innervated by different neurological systems [3]. Photoreceptors in the retina detect and convey the sensory information about retinal illumination to the pretectal olivary nucleus (PON) via optic nerves. The PON synapses at the Edinger Westphal (EW) nucleus [4] which then projects to the ciliary ganglion to control the sphincter muscle via the short ciliary nerve [5, 6]. The neurological pathway related to pupil dilation is still not well understood [5]. The dilator muscle receives control from the superior cervical ganglion via the ciliary nerves. The ciliospinal center of Budge is found to project to the superior cervical ganglion [7].

PLR responses can be altered by dysfunctions in the PLR pathway. In fact, abnormal PLRs have been previously reported in several types of neurological disorders. Fan el al. [8] reported prolonged PLR latency, smaller relative constriction and lower constriction velocity related to autism spectrum disorder (ASD). Giza el al. [9] reported prolonged latency, reduced amplitude, maximum constriction velocity and maximum acceleration associated with Parkinson's disease. Fotiou et al. [10] reported atypical PLR associated with Alzheimer's disease, where all parameters except baseline and minimum pupil diameters were affected.

To develop an effective screen for neurodevelopment disorders, it is important to first understand neurodevelopment in typically developing children. Several studies have been conducted to examine the normal neurodevelopmental progress of the visual system in children by using visual evoked potentials (VEP) [11, 12]. A recent report demonstrated the potential of using PLR to examine visual system development in preterm babies [13]. However, no comprehensive study has been conducted to investigate age related profiles of PLR parameters in children.

Here we report our results of PLR tests in over 100 typically developing children from 6 to 17 years old. Our results revealed a significant age effect in PLR parameters, particularly the PLR latency and resting pupil diameter. A similar trend was not observed in a group of age-match children with neurodevelopmental disorders.

II. Procedure

A. Instrumentation

A custom-built binocular pupilogram recording system (Fig. 1) was used to measure PLR with high spatial (35μm/pixel) and temporal resolution (8.7 ms). The two recording channels are independent but synchronized. The optical stimulation and image acquisition were controlled through a computer interface via a custom-developed Labview program. This customized system has two "sighting" ports so that the participant can fix sight at a given target during PLR test. In addition, this system is versatile for

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setting various stimulation waveforms and intensities.

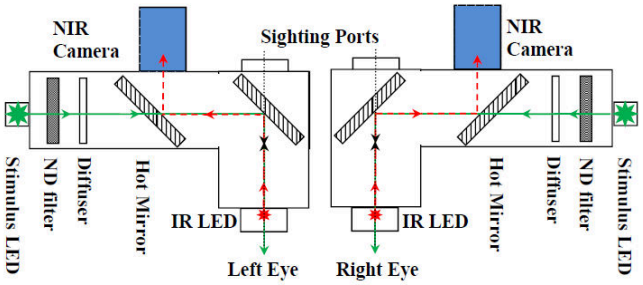

Figure 1. A schematic diagram of the binocular pupilogram recording system. A hot mirror was used in each channel to separate the optical stimulation path and imaging path. The participant can fix the sight on a monitor through the two sighting ports.

Pupils were illuminated by near infrared (NIR) LEDs at 880 nm wavelength. A 530 nm green LED was used to provide the light stimulus for evoking the PLR. The electric current to the LED was controlled to vary the stimulation irradiance along with the use of neutral density (ND) filters. The stimulation light then passed through a diffuser providing on-axis illumination with 5.7° visual field. The stimulation intensities used in this study varied from 0.09 $\mu W/cm^2$ to 9.9 $\mu W/cm^2$ in light-adaptation (LA) and was 0.09 $\mu W/cm^2$ in dark-adaptation (DA).

Two near infrared (NIR) cameras (GC660, Allied Vision Technologies, Stadtroda, Germany) were used in the system to acquire pupil images. The image size was 659 pixels × 494 pixels with a 12 bit resolution. At each PLR test, the cameras were triggered first to acquire baseline pupil images for 1s. Then the green LEDs were triggered to give a 100ms flash. Image acquisition was continued for four more seconds to capture the entire pupil constriction and recovery process. A total of 575 images were acquired from each eye in a single test trial (5 sec). All acquired images were saved using the tiff format.

Custom image processing software developed in visual c++ was used to automatically calculate the pupil diameter from each of the recorded pupil images in the image sequence (575 images for each eye). A histogram-based threshold method was applied after contrast stretching the pupil image to locate boundary pixels for the pupil. The threshold of pupil boundary was identified as the pixel value corresponding to first minima of the image histogram as shown in Fig. 2(b). Using this threshold the images were binarized and pupil was segmented. All pixels on the pupil boundary were then extracted. An ellipse was fitted to the segmented pupil boundary (Fig. 2(a)) by using a direct least square fitting algorithm [14]. The area of the fitted ellipse was used to estimate the pupil area. A nominal diameter was calculated by treating the pupil as a circle.

Once all pupil diameters were extracted from the acquired image sequence, a pupilogram curve (Fig. 3) was constructed to represent the pupil size change in response to the optical stimulus. The pupilogram was normalized against the resting pupil area to remove effects of resting pupil size when calculating constriction amplitude. The following PLR parameters were calculated from the pupilogram in Fig. 3 to quantify the pupillary response. The resting pupil diameter D_0 was calculated by averaging pupil diameters obtained during the 1s period before stimulus onset.

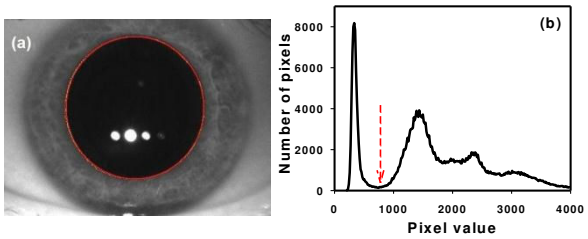

Figure 2. An example to illustrate the pupil segmentation used in our study. (a) An example pupil image. (b)The corresponding histogram. The first minima marked by the arrow in (b) indicates the boundary of the black pupil in (a). This value was used as the threshold to segment the pupil. The red circle in (a) shows the fitted ellipse using least square fitting.

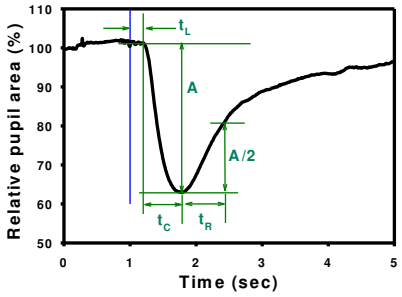

Figure 3. An illustration of the pupilogram which is normalized against the pupil area before stimulus onset (the resting pupil area D_0^2). Following extracted PLR parameters are shown: A= relative constriction amplitude; t_L = latency; t_C = constriction time; t_R= redilation time

The relative constriction amplitude was calculated by normalizing the difference between resting pupil area and minimum pupil area against the resting pupil area. PLR latency (t_L) was calculated as the time interval between stimulus onset and the beginning of pupil constriction. The constriction time (t_C) was calculated as the time interval between the beginning of pupil constriction and when pupil reached minimal size. The redilation time (t_R) was calculated as the time interval between the minimal pupil diameter and when the pupil recovered to half of the constriction. The pupilogram (before normalization) was smoothed by using a 6^{th} order Savitzky-Golay filter. To measure the PLR latency, the acceleration (2^{nd} order derivative) of the pupilogram was calculated. The time of the maximal acceleration was first identified and used as the starting point to back-track toward the stimulation onset. The first image frame that deviated from the baseline pupil size was considered as the onset of pupil constriction.

B. Test procedure

PLR data were obtained in 107 healthy children 6 to 17 years old (mean age 10.9±2.9 years) without any known visual or neurological problems. There were 79 males (mean age 10.9±3.1 years) and 28 females (mean age 10.6±2.4 years). As a comparison, PLR data were also examined in 176 children (mean age 10.5±3.1 years, 150 males and 26 females) with several different types of neurodevelopmental disorders including autism (147), mental retardation or developmental delays (10), Down's syndrome (7), Fragile

X syndrome (5), cognitive disorders (4) , learning disability (1), Prader Willi (1), Oppositional defiant disorder (1). This group of participants was recruited through the Thompson Center for Autism and Neurodevelopmental Disorders at University of Missouri. Written consents were obtained from all participants and their legal guardians as approved by the Institutional Review Board of University of Missouri-Columbia.

PLR was measured in both light adapted (LA) (room luminance of 30cd/m^2) and dark adapted (DA) (<0.02cd/m^2 room luminance) conditions. The intensities used as optical stimulation for PLR were 0.09μW/cm^2 in dark-adaptation, and 0.09μW/cm^2, 1.0μW/cm^2, 9.9μW/cm^2 in light adaptation. For each stimulus condition, PLR responses from both eyes were measured when one eye was stimulated. The measurements were repeated four times for each condition with an approximately 30s interval between two consecutive measurements. Imaging was started 1s before the stimulation to gather the resting pupil size. After the LA test, all participants stayed in the dark room for 15 minutes for the pupils to naturally dilate before starting the DA test.

C. *Data analysis*

The Analysis of Covaraince (ANCOVA) was applied in SAS to examine the effects of age and test conditions on each PLR parameter. Follow up analysis of variance (ANOVA) was performed to verify the age effect for a linear relationship. PLR parameters were verified for normal distribution using the Kolmogorov-Smirnov test. p<0.05 was considered as significant.

III. Results

As expected, in typically developing children the resting pupil diameter was larger in dark adaptation (7.44±0.77 mm) than in light adaptation (6.58±0.61 mm) as shown in Fig. 4. The resting pupil diameter increased with age significantly before 12 years old (F(6,135) = 2.67, p=0.018). From 6 to 12 years old, the mean resting pupil diameter increased 8.0% in LA and 13.2% in DA. The ANOVA test for a linear trend further confirmed that the age effect was significant (p=0.047 at LA and p= 0.003 at DA). At the same stimulus intensity, the PLR constriction amplitude was larger in dark-adaption whereas the constriction/redilation times were longer and latency was shorter. In light-adapted tests, as stimulus intensity increased from 0.09μW/cm^2 to 9.9μW/cm^2, PLR latencies decreased 21.85%; constriction and redilation times increased 25.30% and 48.15% respectively; and relative constriction amplitude increased from 11.76±5.54% to 40.75±7.23%.

The ANCOVA model suggested a significant age effect on several PLR parameters. In children from 6 to 8 years old, the age effect was significant for constriction amplitude (F(3,132)=3.48, p=0.018). PLR constriction increased with age in children younger than 8 years old and reached a plateau thereafter (Fig. 5a) at all stimulation conditions except the one at LA 0.09μW/cm^2. However the linear increasing trend at young age (< 8 years) was significant only with the maximal stimulus at LA 9.9μW/cm2 (F(1,21)=5.70, p=0.027). The PLR constriction time and the redilation time did not show an effect with age.

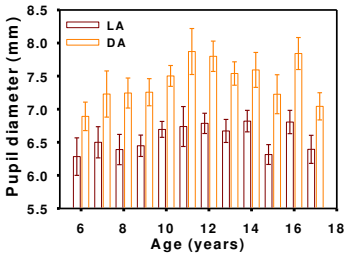

Figure 4. The age effect in resting pupil diameter in the light adapted (LA), and dark adapted (DA) environment in children with typical development. The error bars indicate the standard error.

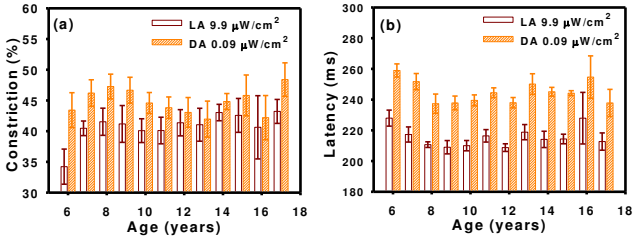

Figure 5. PLR parameters obtained in children with typical development from 6 to 17 years old. (a) Relative constriction amplitude, (b) latency measured in the light adapted (LA) 9.9μW/cm^2 and dark adapted (DA) 0.09μW/cm^2 condition. The error bars indicate the standard error.

The most consistent age effect was observed in PLR latency. The ANCOVA model revealed that in children from 6 to 9 years old, latency has a significant age effect (F(3,132) = 6.68, p<0.001). As shown in Fig. 5b, PLR latency decreased significantly at all testing conditions from 6 to 9 years and stabilized thereafter. For example, the PLR latency decreased from 259.0±4.3ms at 6 years old to 237.3±6.3ms at 8 years old at stimulation condition of LA 9.9μW/cm^2. The ANOVA test for a linear trend in children younger than 10 years old further confirmed that the age effect was significant (p< 0.01) at all conditions except the one with the lowest stimulus intensity of LA 0.09μW/cm^2.

Since we saw a consistently significant age trend in PLR latency, we examined the age trend in PLR latency and resting pupil diameter measured in a group of children of the same age range with neurodevelopment disorders. As shown in Fig. 6, no age dependent trend in PLR latency existed in this group of children. At the same stimulation condition, children with neurodevelopment disorders had significantly longer latency than typically developing children. Similarly we didn't observe any age effects on resting pupil diameter in this group of children.

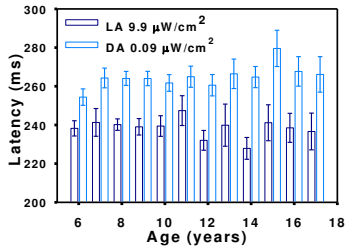

Figure 6. The PLR latency in children with neurodevelopment disorders measured at light adapted (LA) 9.9 μW/cm^2 and dark adapted (DA) 0.09μW/cm^2 condition. The error bars indicate the standard error.

IV. Discussion

PLR is an involuntary neurological response. Testing of PLR is noninvasive, simple and fast. It requires minimal cooperation from the subject and thus is convenient for testing in children. A good understanding of age dependent behavior of PLR is essential to evaluate the potential use of PLR for screening neurodevelopmental disorders in children.

Our results indicated a consistent and statistically significant age effect in PLR latency measured in young children (<10 years old) with typical development. These results appear to be consistent with previous findings of age-dependent changes in visual evoked potential (VEP) in children. Lenassi el al. [12] compared flash VEP and pattern VEP in infants and young children from 1.5 months to 7.5 years of age. They found that VEP latency for all three stimulation types showed an exponential decrease with age, but the trends were different. The latencies of reversal and pattern onset VEP showed fast decays (exponential decay rate of -9.3/year and -13/year respectively) and were stabilized by 6 months of age. However, flash VEP latency showed a slower decay (exponential decay rate of -0.54/year) and still decreased gradually at the upper limit of the age (7.5 years) they tested. Our age-dependent PLR latency in children (6 – 9 years) with typical development had a similar effect as the flash VEP latency results reported by Lenassi el al. [12]. Carrillo-De-La-Pena el al. [11] studied flash VEP in 85 children from 8 -15 years old and reported no significant age effect in latency. This result is consistent with our observation that PLR latency didn't change in children older than 9 years old.

Although a significant age effect was reported in relative constriction, it was statistically significant only at one test condition with the highest stimulus intensity. With a close examination, we noticed that the coefficient of variance for relative constrictions varied from 12% to 63% at those stimulation conditions where the age effect was not statistically significant. At the strongest stimulus of LA at 9.9 $\mu W/cm^2$, the coefficient of variance was much smaller, from 8% to 24%. Hence it is possible that the lack of statistical significance can be attributed to the higher variation in data obtained with smaller stimulus intensities.

The fact that no age-dependent trend in PLR latency or resting pupil diameter was observed in the group of children with neurodevelopment disorders suggests that the typical neurodevelopmental trajectory might be altered in neurodevelopmental disorders. The underlying mechanisms need further study. However, our result suggests that PLR has the potential to provide clinically useful information about progression of neural development in children.

V. Conclusion

We found a significant and consistent age dependent effect in PLR latency in children 6 to 9 years old. We also observed age effects in resting pupil diameter and PLR constriction amplitude. Such an age-dependent effect was not observed in children with neurodevelopment disorders. Further studies in larger groups of children especially in children younger than 6 years old are necessary to fully understand the details of age dependency of PLR. Nevertheless, PLR shows potential to be applied as a simple noninvasive tool to monitor neurodevelopment in children.

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The Neural Basis for Atypical Pupillary Light Response in Autism Spectrum Disorder

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Abstract Text:

Background: Pupillary light reflex (PLR) refers to the involuntary pupillary restriction that is induced by a luminance change. Recent studies (e.g., Daluwatte et al., 2013; Fan et al., 2009) have documented atypical PLR (i.e., longer PLR latency, smaller constriction amplitude, and lower constriction velocity) in individuals with Autism Spectrum Disorder (ASD). The primary neurological pathway subserving the PLR response is well-established and comprises the retina, pretectal nucleus, Edinger-Westphal nucleus, and ciliary ganglion (Lowenstein & Loewenfeld, 1950). There is also evidence, however, of tertiary cortical and cerebellar contributions to PLR (e.g., Tsukahara et al., 1973). Within this context, the neural locus of ASD-related abnormalities in PLR remains unclear.

Objectives: Functional magnetic resonance imaging (fMRI) was used to examine the neural disruption(s) that contribute to atypical PLR in ASD.

Methods: A sample of 25 individuals with ASD (mean age = 16.0 years) and a demographically-matched comparison group of 19 neurologically intact individuals without ASD (mean age = 16.0 years) participated. Scans were obtained on a 3T Siemens Trio scanner with a standard 8-channel head coil. Stimuli were displayed using an LCD projector, and pupillary responses were recorded using a MRI-compatible ASL long range optic eye tracking system. 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 5 s to maintain the interest of the participant. Every 20 s, the participant was presented a green-filtered light stimulus superimposed over the current image for 100 ms. 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 presented over the course of 8 functional MRI runs, each of which lasted approximately 4 1/2 minutes.

Results: As anticipated, both groups showed robust PLR-related activation in primary visual sensory areas including lateral geniculate nucleus [$F(1,40) = 16.3$, $p < .0005$] and striate cortex [$F(1,40) = 17.8$, $p < .0005$]. PLR-related activation was also observed in association areas including superior parietal cortex [$F(1,40) = 17.4$, $p < .0005$] and right lateral prefrontal cortex [$F(1,40) = 9.0$, $p < .05$]. Most importantly, group differences in PLR-related activation were evident in the cerebellum as well as anterior insula and superior frontal gyrus, [$F(1,40) > 20$, $p < .00005$ in all instances].

Conclusions: These results are consistent with the hypothesis that prolonged PLR latency observed in individuals with ASD is associated with cerebellar and prefrontal dysfunction.

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Association between Sensory Processing and Pupillary Light Reflex in Children with Autism Spectrum Disorders

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Background: Atypical pupillary light reflexes (PLR) have been observed in children with autism spectrum disorders (ASD), suggests potential autonomic nervous system (ANS) dysfunction in ASD. ANS is also involved in modulating sensory processing and sensory abnormality was widely reported in children with ASD. However, the potential correlation between physical measurements (e.g. PLR) and behavioral observations (e.g. sensory) has rarely been examined in literature.

Objectives: To study the association between sensory behavior and PLR parameters in children with ASD.

Methods: We examined PLR in 259 children including 152 with ASD (age 10.7±3.4 years) and 107 with typical development (TD) (age 10.9 ± 2.9 years). The test was conducted in both light adapted (LA) and dark adapted (DA) conditions using a two channel binocular apparatus. To quantify PLR responses, five basic PLR parameters were extracted including resting pupil diameter, relative constriction, latency, constriction velocity and redilation velocity. The parent or guardian of the participant completed a 29-item questionnaire that was designed to evaluate sensory behavior. Linear correlations were first applied to analyze the association between PLR parameters and sensory scores. Linear regression was then used to investigate whether variations in PLR parameters can be explained by a combination of sensory symptoms. The partial least squares (PLS) regression was also performed to select a subset of sensory behavior as predictor variables that can explain the maximum variance in PLR parameters.

Results: PLR constriction was correlated with the total sensory score in all LA tests ($r \approx -0.3$, $p < 0.05$) in the ASD group but not in typically developing children ($p > 0.05$). No correlation was found between sensory score and other PLR parameters. PLR constriction obtained in the ASD group at the highest stimulus intensity in light adaptation was best predicted in our regression analysis using the sensory item "Avoids getting messy" ($b = 1.4$ $p=0.017$) and "Difficulty paying attention" ($b = 1.8$ $p=0.005$). Post-hoc one-way ANOVA revealed significant effects from items "Avoids getting messy" ($F=4.93$ $p=0.028$) and "Difficulty paying attention" ($F=7.05$ $p=0.0088$) on PLR constriction in the ASD group. Children with ASD who reported "rarely" or "never" on the aforementioned two items had higher PLR constriction than those who reported "always". In the PLS regression model, the above two items plus 7 others were selected and can explain 11.1% of the data variance in PLR constriction.

Conclusions: A weak but significant correlation existed between PLR constriction and sensory score in the ASD group but not in typically developing children. A lower PLR constriction

suggests lower parasympathetic modulation. This observation implied that the abnormal sensory behavior in children with ASD could be associated with lower parasympathetic modulation.

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Pupillary Light Reflex Parameter Constriction Amplitude Relationship to Clinical Symptoms

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Background: Pupillary light reflex (PLR) provides a non-invasive model system for study of the nervous system in ASD. Pupillary response to a light flash includes a latency period, then pupil constriction and recovery. Daluwatte et al, (2012) found PLR parameters discriminated ASD children from typical developing (TD) controls with 87.7% specificity and 76.4% sensitivity. Four PLR measurements (constriction amplitude (CA), latency, constriction time, redilation time) were calculated to quantify PLR. Though latency was the strongest ASD predictor, each parameter strengthened the association. To determine significance of each parameter and to assess their potential usefulness as ASD biomarkers, we have undertaken an analysis of associations between PLR parameters and ASD symptoms. Constriction amplitude (CA) assesses autonomic function, since the constriction sphincter is innervated by the parasympathetic system. A small but consistent literature finds that children with autism have lower parasympathetic and higher sympathetic activity. Moreover, children with ASD commonly present with GI, urinary, sensory, sleep disturbances which are to some degree under ANS control. We question whether variation in CA might inform us about systemic ANS dysfunction.

Objective: Investigate the association between CA and clinical ASD symptoms.

Methods: PLR was measured in 152 children with ASD (age 10.7±3.4 years, 135 males and 17 females) and 107 TD children (age 10.9 ± 2.9 years, 79 males and 28 females). PLR induced by a 100ms green light was measured in light adapted and dark adapted conditions using a two channel binocular apparatus. PLR measurements were calculated to quantify PLR. A parent questionnaire designed to evaluate areas of ANS participation, including GI, GU, fever response, sleep and hyper-sensitivity was completed. From this group, 53 ASD children who had also completed the Simons Simplex Study were selected for preliminary analysis.

Results: CA was in autism range or below for 53%, in normal range for 34% and equivocal for 13%. Using CA as dependent variable, we found low CA correlated significantly with lower IQ (FSIQ & NVIQ; p= 0.02, VIQ: p=0.03). In addition, children with the lowest CA were more than twice as likely to have parents report improvement in core ASD symptoms with fever (40% vs19%). This confirmed findings from our initial PLR subjects (Fan et al., 2009). Systemic symptoms influenced by the ANS (GI, sensory, sweating, salivation, urination, swallowing, sleep disturbances) were not associated with CA. Though not reaching significance the low CA group showed a trend toward further neurologic dysfunction based on higher toe walking and dysmorphology.

Conclusions: PLR CA is a measure of ANS activity. Our data show CA correlates inversely with heart rate, indicating low pupillary CA could be an indicator of systemic ANS dysfunction in ASD. In this small sample none of the clinical symptoms suggestive of ANS dysfunction correlate with CA. We did find children with the smallest CA had significantly lower IQs suggesting variation in CA may be a marker for general neurologic disruption. Clarification of these results will depend on our ongoing analysis in the entire sample of 152 ASD children and 107 TD children.

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ASSOCIATION BETWEEN PUPILLARY LIGHT REFLEX AND SENSORY BEHAVIOR IN CHILDREN WITH AUTISM SPECTRUM DISORDER

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OBJECTIVES: Pupillary light reflex (PLR) is a simple noninvasive neurological test that can reveal a rich set of neurological information. Sensory Profile questionnaire is widely used in clinic to evaluate sensory processing in children. Both atypical PLR and sensory processing have been reported in children with autism spectrum disorder (ASD). We investigated here whether the physical PLR measures are correlated with the behavioral sensory measures.

METHODS: We measured PLR and sensory profile in 152 children (10.7 ± 3.4 years old) with ASD and 107 children (10.9 ± 2.9 years old) with typically development (TD) between 6-17 years. PLR was measured in both light and dark adaptations and quantified using 5 parameters: resting pupil diameter, relative constriction, latency, constriction time and redilation time. Participants' parents completed 29 sub-items from the Sensory Profile questionnaire. Associations among PLR parameters and sensory scores were first analyzed using linear correlations. Partial least squares (PLS) regression was then performed to select sensory items which are best associated with PLR parameters.

RESULTS: A significant correlation was found between PLR constriction and the sensory total score in the ASD group ($r \approx 0.3$, $p < 0.02$) but not in TD group ($p > 0.05$). PLS regression selected 9 sensory items associated with PLR constriction. Children with ASD who reported "always" on these sensory behaviors had lower PLR constriction than those who reported "never".

DISCUSSION/SIGNIFICANCE OF IMPACT: This is the first study that revealed association between PLR and clinical behavioral assessment. A smaller PLR constriction suggests less parasympathetic modulation. Thus our results implied that abnormal sensory behavior in children with ASD could be associated with lower parasympathetic modulation.

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Atypical pupillary light reflex and heart rate variability in children with autism

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Background: Atypical pupillary light reflexes (PLR) were previously reported in children with Autism Spectrum Disorder (ASD). A replication study is being conducted in a larger population to further investigate PLR profiles in children with ASD. Heart rate variability (HRV) was also measured simultaneously to explore potential impairments in the autonomic nervous system (ANS) associated with ASD.

Objectives: To study PLR and HRV profiles in children with ASD.

Methods: PLR and HRV were analyzed in 143 children with ASD (age 10.7±3.4 years, 128 males and 15 females) and 109 children of typical development (age 11.0±2.9 years, 80 males and 29 females). PLR induced by a 100ms green light was measured in both light adapted (LA) and dark adapted (DA) conditions using a two channel binocular apparatus. Five basic PLR measurements including resting pupil diameter, relative constriction, latency, constriction velocity and redilation velocity were calculated to quantify PLR. HRV was measured using a remote heart rate device during the entire PLR test. In addition to time domain HRV parameters, Fourier transform was applied to calculate the high frequency ("HF") and low frequency ("LF") components of the RR tachogram power spectrum.

Results: Similar to the previous findings, children with an ASD had significantly longer PLR latency ($p < 0.0001$) and smaller PLR constriction ($p = 0.0034$) than the typical controls. In typical controls, the PLR latency decreased significantly from 6 to 8 years old (one way ANOVA $p < 0.05$) and stabilized thereafter. No significant age effect was observed in latency obtained in the ASD group. The average heart rate was significantly higher in children with an ASD ($p < 0.05$). The control group showed lower normalized HF power (high frequency power divided by total of high frequency and low frequency power) and higher LF/HF ratios (ratio between high frequency power and low frequency power) during the PLR test than during the resting periods ($p < 0.05$). The same change was also observed in the ASD group, but the magnitude of change was much smaller than that of the controls.

Conclusions: The atypical PLR profiles found in our preliminary study were confirmed in a larger ASD population in this study. The different age effect on PLR latency suggests that the developmental trajectory associated with PLR pathway may be altered in children with ASD. The observed high average heart rate indicated elevated sympathetic tone in the ASD group. HRV changes during administration of the PLR (higher LF/HF and lower HF power) suggest that children with ASD have an altered ANS response to the PLR.

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Simultaneous measurement of pupillary light reflex and heart rate variability in children with autism

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Background: Atypical pupillary light reflexes (PLR) were recently observed in children with Autism Spectrum Disorder (ASD). Studying simultaneous heart rate variability (HRV) would provide insights into associated non-specific impairments in the autonomic nervous system (ANS).

Objectives: To investigate the association between atypical PLR parameters and non-specific impairments in ANS by measurement of heart rate variability simultaneously with PLR in children with ASD.

Methods: PLR is measured using a two channel binocular apparatus, and HRV was measured using a remote heart rate device. PLR was induced by a 100ms green light pulse and measured in both light adapted (LA) and dark adapted (DA) conditions. Heart rate recording was started five minutes before the PLR test while keeping the participant in a sitting position and ended five minutes after the test. The tests were conducted in 71 children with ASD (age 11.3±3.0 years, 63 males and 8 females) and a typically developing control group of 50 children (age 10.8±2.4 years, 26 males and 24 females). To study medication effects, the ASD group was divided into a medication group (if the participants were taking antipsychotics, ADHD medications, antidepressants, etc.) and a non-medication group. Five basic PLR measurements including resting pupil diameter, relative constriction, latency, constriction velocity and re-dilation velocity were calculated to quantify PLR. To analyze heart rate variability, Fourier transform was applied to calculate the high frequency (0.15 – 0.4 Hz, "HF") and low frequency (0.04-0.15Hz, "LF") components of the RR tachogram power spectrum.

Results: Similar to the previous findings, PLR latency was significantly longer in children with an ASD than children of typical development ($p < 0.0001$). The ASD group with medications had smaller PLR constriction than the typical controls and the non-medication ASD group ($p < 0.001$). The latency difference between the ASD group with and without medication was not significant. The average heart rate was significantly higher in children with an ASD ($p < 0.05$). Both the ASD and control groups showed smaller normalized HF power and higher LF/HF ratios during the PLR test than during the resting periods ($p < 0.05$). However such PLR test associated changes were significantly smaller in the ASD group than in the typically developing control group.

Conclusions: The atypical PLR profiles found in our preliminary study were confirmed in the larger ASD population tested in this study. The observed high average heart rate suggested an increased sympathetic tone in the ASD group. Our results also indicated that PLR testing itself induced less ANS modulation changes in the ASD group than in the typical controls.