ARTICLE

# Altered pupil responses to social and non-social stimuli in *Shank3* mutant dogs

Wei Ren[1,2], Kang Huang[3], Yumo Li[1,4], Qin Yang[5], Liping Wang[2,5], Kun Guo[6]✉, Pengfei Wei[2,5]✉ and Yong Q. Zhang[1,2,7]✉



Pupillary response, an important process in visual perception and social and emotional cognition, has been widely studied for understanding the neural mechanisms of neuropsychiatric disorders. However, there have been few studies on pupil response to social and non-social stimuli in animal models of neurodevelopmental disorders including autism spectrum disorder (ASD) and attention deficit hyperactivity disorder. Here, we developed a pupilometer using a robust eye feature-detection algorithm for real-time pupillometry in dogs. In a pilot study, we found that a brief light flash induced a less-pronounced and slower pupil dilation response in gene-edited dogs carrying mutations in *Shank3*; mutations of its ortholog in humans were repeatedly identified in ASD patients. We further found that obnoxious, loud firecracker sound of 120 dB induced a stronger and longer pupil dilation response in *Shank3* mutant dogs, whereas a high reward food induced a weaker pupillary response in *Shank3* mutants than in wild-type control dogs. In addition, we found that *Shank3* mutants showed compromised pupillary synchrony during dog-human interaction. These findings of altered pupil response in *Shank3* mutant dogs recapitulate the altered sensory responses in ASD patients. Thus, this study demonstrates the validity and value of the pupilometer for dogs, and provides an effective paradigm for studying the underlying neural mechanisms of ASD and potentially other psychiatric disorders.

*Molecular Psychiatry* (2023) 28:3751–3759; https://doi.org/10.1038/s41380-023-02277-8

## INTRODUCTION

Pupillary responses are widely used as an informative parameter to evaluate arousal and emotional state, and cognitive processing such as visual attention and memory [1–3]. Pupillometry has the potential to assist in diagnosis of psychiatric disorders [4] such as autism [5, 6], anxiety [7, 8], and depression [9, 10]. Compared with typically developed children, autistic children show significantly longer pupillary light reflex latency, smaller constriction amplitude and slower constriction velocity [11, 12]. Pupillary responses in autistic children are also hypersensitive to repeated sounds [13]. However, no specific genetic defects associated with abnormal pupillary responses have been identified in these patients.

Pupillary responses have also been used to examine arousal state and sensory and cognitive processing in animals, including rodents [14] and non-human primates [15]. They are a useful measure for exploring human neuropsychiatric disorders, such as autism spectrum disorder (ASD), in animal models. For instance, compared with wild-type (WT) rodents, prolonged pupil response and larger pupil size occur in CDKL5- and MeCP2-deficient mouse models of ASD [16], and the latency of pupillary light reflex was increased in an ASD model of macaques carrying *SHANK3* mutations [17]. However, the inherent constraints of these animal models may limit their implications in studying ASD. Specifically, although genetically-modified rodents are the animal models most commonly used to dissect the molecular and circuitry mechanisms underlying ASD, the apparent differences in brain anatomy and social behavior between rodents and humans may limit the translational value of rodent models for studying ASD. Non-human primate models could provide insights into the pathophysiology of ASD, but the slow reproduction rate (e.g., 5 years to reach adulthood and one birth per pregnancy) and extremely high cost pose a practical challenge for their broad application [18–20].

Among the myriad species on earth, dogs share human physical and social environments, and they have developed complex social interactions with humans over 33,000 years of domestication [21, 22]. Genome comparison revealed that the dog genome closely resembles the human genome [23]. Proteomics analysis showed more conserved spatiotemporal protein expression patterns in dog brains compared to human brains than those of mouse brains [24]. Furthermore, behavior studies have revealed that dog-human relationship plays a crucial role in the social behavior of dogs [25], and they display human-like gaze allocation or behavior in viewing social scenes [26] and in emotional connection with humans [27]. Thus dogs are an effective animal model for studying social cognition and neuropsychiatric disorders, such as ASD in humans [28–30].

Although a pupillometry system has been used to study dog cognition [31], this setup restricts head and body movements,

[1]State Key Laboratory for Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China. [2]College of Life Sciences, University of Chinese Academy of Sciences, Beijing 100049, China. [3]Shenzhen Bayone BioTech Co. Ltd, Shenzhen 518100, China. [4]College of Life Sciences, Beijing Normal University, Beijing 100875, China. [5]Shenzhen Key Lab of Neuropsychiatric Modulation and Collaborative Innovation Center for Brain Science, Guangdong Provincial Key Laboratory of Brain Connectome and Behavior, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, and Shenzhen-Hong Kong Institute of Brain Science-Shenzhen Fundamental Research Institutions, Shenzhen 518055, China. [6]School of Psychology, University of Lincoln, Brayford Pool, Lincoln LN6 7TS, UK. [7]School of Life Sciences, Hubei University, Wuhan 430415, China. ✉email: kguo@lincoln.ac.uk; pf.wei@siat.ac.cn; yqzhang@genetics.ac.cn

Received: 11 March 2023 Revised: 21 June 2023 Accepted: 14 September 2023
Published online: 17 October 2023

leading to difficulties in studying naturalistic behavior and social interaction which are crucial for understanding ASD. In addition to the challenge of establishing the eye-tracker on the head for reliable prolonged data collection, another difficulty is that unlike humans, dogs have a reflective lining behind the retina, the tapetum lucidum, which reflects part of the light back to the camera, producing a bright pupil to obstruct precise measuring of pupil size [32]. Existing pupil segmentation algorithms are not able to resolve the bright pupil effect, leading to inaccurate data collection and analysis.

Therefore, we set out to develop a robust head-mounted pupillometry system for dogs based on machine learning. This system not only improves data collection quality and data analysis accuracy, but also allows for real-time pupil size measurement. Furthermore, it allows multiple pupillometry systems to work simultaneously, facilitating the study of naturalistic social interactions among multiple individuals. In the present study, we validated and used this newly developed pupillometry system to determine whether ASD-associated genes affect pupillary responses in dogs. We systematically compared differences in pupillary responses to a range of non-social (light, sound, and food) and social (dog-human interaction) stimuli between dogs with and without *Shank3* mutations; *SHANK3* mutations are well-known genetic risk factors for ASD in humans [33].

## MATERIALS AND METHODS

### Ethical consideration and animal care

All animal-related protocols were approved in advance by the Animal Care and Use Committee of the Institute of Genetics and Developmental Biology (AP2022001). Three control Beagle dogs (1–2 years old, male; provided by Beijing Sinogene Biotechnology Co. Ltd) and three heterozygous *Shank3* mutants (−1279 + 1 bp/+, −496 bp/+ and −483 + 7 bp/+, 1–2 years old, male) previously generated and characterized [29] were used for various experiments in the present study. The dogs were housed in pairs after weaning at postnatal day 50 in 2 m × 0.9 m × 1.5 m (length × width × height) cages and maintained on a 12/12 h dark light cycle, with a humidity of 40–60% and a temperature of 22–24 °C. During non-experimental periods, dogs were exposed to the breeders only. The dogs were fed with Royal Canine Chow (Royal Pet Food Company Ltd., France) twice daily from 08:00 to 10:00 and 15:30–17:00. Based on regular veterinary assessments, all dogs were in good health at the time of experiments. All tests were performed at the timeslot of 9:00–12:00 or 14:30–17:00.

### Pupillometry after non-social and social stimuli

Before the experiment, each dog was guided into an area (2 × 3 m) surrounded by fences, and allowed to explore freely for 10 min. Dogs first completed fitting training of the pupillometer for 8 sessions per day, starting with the loosest setting and leaving it on for less than 10 s. We adjusted the fit of the pupillometer to be snug on dogs' heads and gradually let them wear for a longer period of time. Approximately three days after the fitting training, dogs were qualified to complete the following tests if they were able to wear the pupillometer comfortably for 10 min. Light conditions in the room were kept constantly at 90 lux using LED-light bulbs (10 W, 4000k, Philips, Netherland) except for light stimuli experiments.

When recording pupillary responses to light stimuli, dogs experienced dark adaptation (40 lux) for 5 min before the light stimulus. The light source was located 10 cm in front of the dogs. The light stimulus (1000 lux, 630 nm) was presented for 0.5 s. Each dog completed three sessions, consisting of 5 trials per session per day. The inter-trial interval was 5 min (dark adaptation).

When recording pupillary responses to auditory stimuli, the loud auditory stimuli of 120 dB were generated by an electronic firecracker (B806-4YX, BANGREN, China) which was placed at a distance of 1.5 m from the dog. The mild auditory stimulation of 80 dB firecracker sounds was generated by a speaker (B08SXPH879, Bang & Olufsen, Danmark) which was also placed at a distance of 1.5 m from the dog. Every sound stimulus lasted for 200 ms. Each dog completed three sessions, consisting of 5 trials per session per day with a 3 min inter-trial interval.

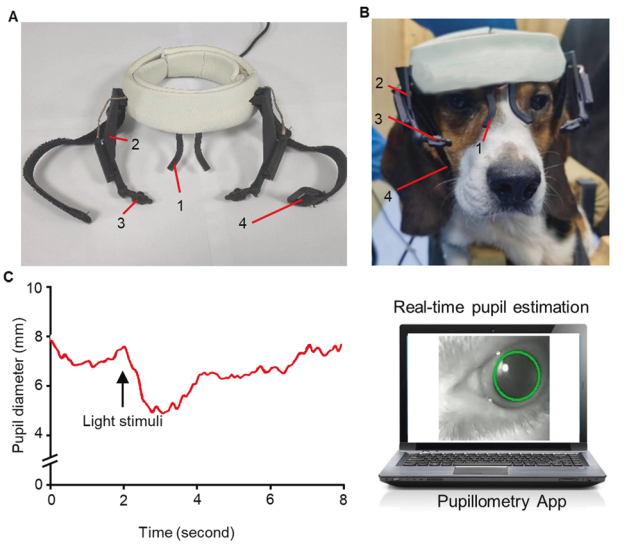


**Fig. 1 Design of a head-mounted and real-time pupillometry system for dogs. A** Key parts of the pupillometer. 1, head-fitted frames; 2, adjustable frames; 3, eye camera; and 4, elastic band. **B** The pupillometer when worn on. **C** Real-time recording of pupil size. Black arrow indicates stimulus onset.

When recording pupillary responses to food stimuli, we delivered high-reward food (snack; BG-W147, Luanhu, China) and low-reward regular food (Royal Canine Chow, Royal pet food company limited, France) separately to the dogs through a custom-made treat dispenser (EV160, EVNICE, China) with a 16-treat capacity, which was driven by a stepper motor drive and controlled by a custom-written python script. All the tested dogs were food restricted overnight for 12 h to ensure their desire for food. Sitting in front of the dispenser, each dog completed three sessions of food stimuli experiments, consisting of 5 trials per session per day with a 3 min inter-trial interval.

When recording pupillary responses during dog-human interaction, the dog and the familiar experimenter stayed in the same room without mutual gaze and no petting at the first stage. They then interacted with each other by petting the dog neck or back and mutual gaze at the second stage for 30 s. Every dog completed three sessions of the assay (five trials per session per day). The inter-trial interval was 3 min. Pupillary responses were recorded for 30 s at each stage of the assay.

Animals of each group were randomly selected and tested. The investigator was not blinded to the genotypes of animals during the experiments and also during outcome assessment. More details on mutant dogs, performance comparison of different pupil detection methods, development of pupillometry, pupil detection algorithm, and determination of pupil dilation synchrony are provided in supplemental methods.

### Quantification and statistical analysis

Statistical analysis was performed using GraphPad Prism (www.graphpad.com). We used non-parametric tests that are more appropriate for small sample sizes and do not require the assumption of equal variances. Inter-group differences between pupil diameters of WT and mutants were analyzed by two-sided Kruskal-Wallis test, while intra-group differences in WT or mutants were analyzed by two-sided Mann-Whitney test. Differences between paired pupil diameters in response to low- and high-reward food stimuli were analyzed by two-sided Wilcoxon test. All samples/animals were included in the analysis. The sample size was not pre-determined. Significance was set to be $p < 0.05$, adjusted for multiple comparisons by the Holm-Šídák method.

## RESULTS

### Light flashes induced slower and reduced pupil constriction in *Shank3* mutant dogs

To examine pupillary responses in dogs, we developed a head-mounted eye tracker capable of artificial intelligence-assisted measuring of real-time pupil size (Fig. 1 and supplementary video). In comparison with other head-mounted eye trackers for dogs

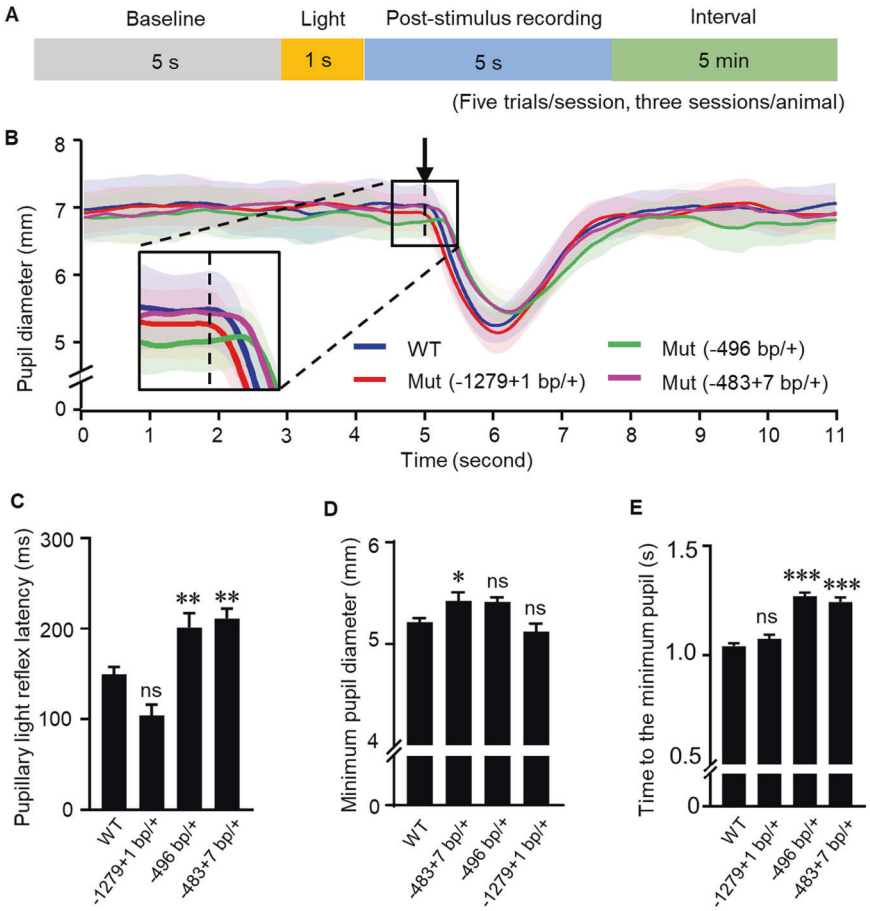


**Fig. 2 Brief light flash stimuli induce a slower and reduced pupil constriction response in *Shank3* mutant dogs. A** A short light stimulus paradigm includes three blocks: grey = baseline recording of pupil diameter; orange = light stimulus; blue = post-stimulus recording; green = inter-stimulus interval. 15 stimuli for each animal. **B** Pupil diameter as a function of time in response to light stimuli in wild-type and *Shank3* mutant dogs (color-coded). WT, wild type; Mut, mutant. Black arrow indicates stimulus onset. The −1279 + 1 bp/+ mutant displayed a stronger pupillary response than the other two mutants. **C** Pupil light reflex latency after light on in wild-type and *Shank3* mutant dogs. Error bars represent standard error of mean (SEM). $^{**}p < 0.01$; ns, no significance compared with WT controls by Kruskal-Wallis test. **D** Minimum value of pupil diameter after light on in wild-type and *Shank3* mutant dogs. Error bars represent SEM. $^{*}p < 0.05$ (Kruskal-Wallis test). **E** Time taken for constriction to the minimum pupil diameter in wild-type and *Shank3* mutant dogs. Error bars represent SEM. $^{***}p < 0.001$ (Kruskal-Wallis test).

[32, 34], our system has the advantages of a lightweight design (53 g for ours vs. 612 g for the other [34]) and a higher frame rate (120 Hz for ours vs. 29.92 Hz for the other [34]) for the eye camera, and a reduced training/habituating time. To compare the performance of different pupil detection methods, we tested our algorithm against 10 commonly-used pupil extraction methods, including two traditional image segmentation methods: ROI-Seg and adaptive threshold segmentation (AdaThresh) [35]; and seven deep neural network-based semantic segmentation methods: FCN [36], Unet [37], ResNet18 [38], ResNet50 [38], InceptionResNetV2 [39], MobileNetV2 [40], Xception [41]; and DLC-based pupil landmark estimation [42]. The systematic comparison of pupil extraction methods indicated that our method performed better in terms of accuracy (the mean absolute error was used as the metric for accuracy evaluation, see Supplementary Table).

Pupillary light reflex refers to pupil constriction after light stimulation. After a brief light flash, we observed pupil constriction in WT control dogs as expected (Fig. 2A and B). To examine if *Shank3* gene mutations affected pupillary light reflex, we analyzed pupil responses in three lines of mutant dogs carrying heterozygous *Shank3* mutations (−483 + 7 bp/+, −496 bp/+, and −1279 + 1 bp/+ with DNA indels in coding region) generated by a protocol of CRISPR/Cas9 gene editing [29]. The *Shank3* mutants exhibited distinct and robust social behavior deficits including social withdrawal in three-chamber test and reduced cross-species social interactions with humans [29], reminisent of that in autistic patients. After a transient light flash of 1000 lux, the pupillary light reflex latency (from the onset of light stimulus to the start of pupil constriction) was 149.60 ± 8.27 ms in WT dogs, but 211.10 ± 11.11 ($p < 0.01$), 201.10 ± 15.98 ($p < 0.01$) and 104.00 ± 12.00 ($p > 0.05$) ms in −483 + 7 bp/+, −496 bp/+, and −1279 + 1 bp/+ mutants, respectively (Fig. 2C). The increased pupillary light reflex latency in *Shank3* mutants indicates impaired neural signal transduction efficiency, as suggested previously [43]. The minimum pupil diameter was 5.22 ± 0.04 mm for WT dogs, but 5.43 ± 0.08 ($p < 0.05$), 5.41 ± 0.05 ($p > 0.05$) and 5.12 ± 0.08 ($p > 0.05$) mm for −483 + 7 bp/+, −496 bp/+ and −1279 + 1 bp/+ mutants, respectively (Fig. 2D); the average time taken for constriction to the minimum pupil diameter was 1.04 ± 0.01 s for WT, but 1.24 ± 0.02 ($p < 0.001$), 1.27 ± 0.02 ($p < 0.001$) and 1.08 ± 0.02 s ($p > 0.05$) for −483 + 7 bp/+, −496 bp/+, and −1279 + 1 bp/+ mutants, respectively (Fig. 2E), indicating slower and weaker pupil constriction after light stimulation in *Shank3* mutant dogs. The weaker phenotype in −1279 + 1 bp/+ mutant compared with −483 + 7 bp/+ and −496 bp/+ mutants is consistent with the fact that −1279 + 1 bp mutation leads to an in-frame deletion (a weaker mutant allele) while −483 + 7 bp and −496 bp result in frame shift and truncated proteins (stronger mutant alleles) [29].

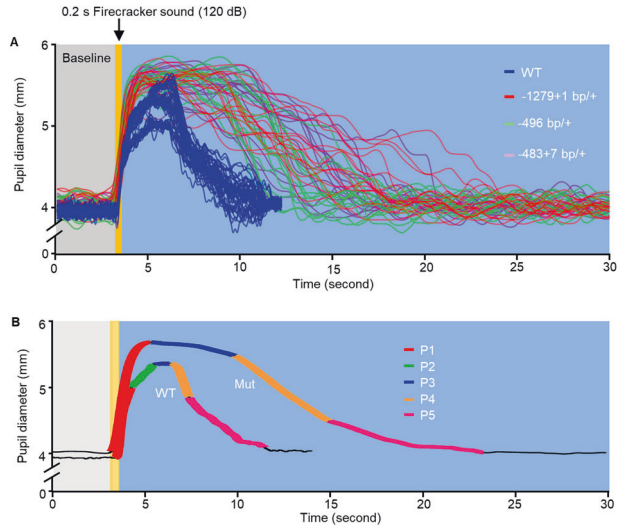


**Fig. 3 A loud (120 dB) firecracker sound induces elevated and sustained pupil dilation in *Shank3* mutant dogs. A** Pupil diameter as a function of time following a 120 dB sound stimulus. WT, wild type; Mut, mutant. 15 stimuli for each animal. **B** Mean pupil diameter as a function of time following a 120 dB sound stimulation in wild-type and *Shank3* mutant dogs. The profile of pupil size changes after sound stimuli in WT controls is divided into five phases (color-coded) by changing slopes. P1−P5: phases 1 to 5. WT, wild type; Mut, mutant.

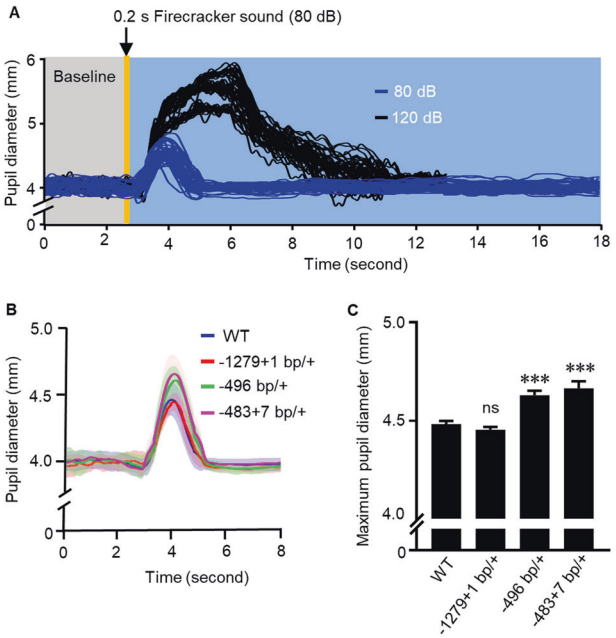


**Fig. 4 A mild auditory stimulus induces a slightly enhanced pupil dilation response in *Shank3* mutant dogs. A** Pupil diameter as a function of time in response to 80 dB and 120 dB sound stimuli (color-coded). Black arrow indicates the sound stimulus onset. **B** Pupil diameter as a function of time following 80 dB sound stimuli. Mutants are color-coded. WT, wild type. 15 stimuli for each animal. **C** Maximum value of pupil diameter after 80 dB stimulation in WT and *Shank3* mutant dogs. Error bars represent SEM. ***$p < 0.001$; ns, no significance compared with WT controls (Kruskal-Wallis test).

### Loud 120 dB firecracker sound induced elevated and sustained pupil dilation in *Shank3* mutant dogs

Abnormal responses to sensory inputs, such as sounds, are one of the core symptoms of autism [44]. Around 50−70% of autistic children and adults show decreased tolerance to environmental sounds [45], which is a cause of significant distress and inconvenience for them, leading to anxiety, reduced community involvement, and school/workplace difficulties [46, 47]. As dogs are typically fearful of firecracker sounds [48], we delivered a 120 dB firecracker sound as an aversive auditory stimulus to examine possible abnormal pupil responses in *Shank3* mutant dogs. Compared with WT dogs, pupil dilation in mutants was faster to the maximum diameter and more elongated (i.e., it took longer to recover to baseline). Specifically, pupils of WT dogs dilated to the maximum diameter in 2.8 s after the 120 dB firecracker sound and gradually recovered to baseline over the next 4.9 s, whereas pupils of mutants dilated to the maximum diameter in 2.0 s and recovered over the next 16.8 s (Fig. 3A). Notably, WT dogs showed individual differences in pupillary responses to the sound stimuli, but the mutants did not.

A schematic comparison of pupillary responses between WT and mutant dogs is presented in Fig. 3B. Pupillary responses in WT dogs could be divided into five temporal phases (P1, rapid phase of dilation, P2, sustained phase of dilation, P3, plateau phase of dilation, P4, rapid phase of constriction, P5, slow phase of constriction) based on the observed boundary points and the significant differences in slope between consecutive phases ($p < 0.001$, Mann-Whitley test). Mutants, however, lacked P2 and displayed a much longer P3 and a lower constriction rate of P4 and P5 (Fig. 3B).

### A mild firecracker sound induced enhanced pupil dilation in *Shank3* mutant dogs

Given that sounds of different loudness may cause different levels of emotional arousal, and since pupil dilation upon an auditory stimulus is positively correlated with loudness [49], we further examined whether the abnormal pupillary responses of the mutants could be affected by the intensity of auditory stimuli. A mild 80 dB firecracker sound was delivered using an identical experimental protocol to that used for 120 dB sound. The 80 dB and 120 dB firecracker had identical sound properties (tone and timbre) except for amplitude (sound pressure). Overall, 80 dB sound induced a weaker dilation response than 120 dB sound in WT dogs (Fig. 4A). The mean maximum diameters of pupil dilation induced by 80 and 120 dB sounds was 4.48 and 5.44 mm (a difference of 21%), respectively (Fig. 4A). As expected, there were clear differences in pupil dilation responses to 80 dB firecracker sound between WT and mutants (Fig. 4B, C). The maximum pupil diameter was 4.48 ± 0.02 mm for WT, but 4.67 ± 0.04, 4.63 ± 0.02 and 4.45 ± 0.01 mm for −483 + 7 bp/+, −496 bp/+ and −1279 + 1 bp/+, respectively); except for −1279 + 1 bp/+, the mutants of −483 + 7 bp/+ and −496 bp/+ exhibited significantly stronger pupil dilation responses compared with WT dogs ($p < 0.001$; Fig. 4C).

### Pupillary responses in *Shank3* mutants were not sensitive to food reward valence

In addition to auditory stimuli, visual and olfactory cues are also important sensory inputs for cognitive processing in dogs. When viewing food stimuli, ASD patients showed increased activation in the anterior cingulate cortex and either bilateral insula [50] or left anterior insula [51] compared to a control group. However, the etiology of ASD patients was not identified. Here we used both high- (treat) and low-reward (regular) foods to examine whether dog pupillary responses to the integrated visual-olfactory food stimuli could be modulated by the perceived differences in the level of reward, and whether dogs with the ASD-associated *Shank3* mutations displayed atypical pupillary responses to food stimuli (Fig. 5A). Although both WT and mutant dogs showed pupil dilation to food stimuli at different valences, the 483 + 7 bp/+ and −496 bp/+ but not −1279 + 1 bp/+ heterozygotes showed a significantly reduced pupil dilation in terms of maximum pupil

diameter in response to high-reward food compared with WT controls (Fig. 5B, C). Interestingly, WT and mutant dogs tended to respond differently to the reward level. In comparison with a low-reward food, a high-reward food induced significantly larger pupils in WT control and −1279 + 1 bp/+ mutant dogs, but not in −483 + 7 bp/+ and −496 bp/+ mutant heterozygotes (Fig. 5D), indicating that *Shank3* mutants are not sensitive to food stimuli at different reward levels.

### Loss of pupil dilation synchrony in *Shank3* mutants during dog-human interactions

Persistent deficits in social communication and social interaction across multiple contexts are core ASD symptoms [44]. During normal human social interaction, the ability to attend to the same information as another person enables communication and connection, transfer of information and experience sharing [52, 53]. The physiological synchrony associated with shared attention has been supported by several lines of evidence, including pupillary synchrony during human-human interactions [54]. To determine whether pupillary synchrony could be extended to human-dog dyads, and whether mutant dogs exhibited atypical human-dog pupillary synchrony, we quantified the dynamics of pupil dilation in the interacting human-dog dyads (Fig. 6A). We designed a two-stage test for this purpose. For non-interaction control (first stage), the dog and the familiar experimenter stayed in the same room without petting and mutual gaze. For social interaction (second stage) the experimenter interacted with the dog by mutual gaze and petting the dog's neck or back. We analyzed pupil synchronization by scoring pupil dilation as "1" and others as "0" (Fig. 6B), which produced binary vectors consisting of 1 and 0. The magnitude and the frequency of pupil dilation were not considered in this algorithm. We then analyzed pupil dilation between dog and human by calculating the Jarccard distance i.e., the synchronization index between binary vectors (see Supplementary Information). The results showed that the synchrony index of pupillary dilation in WT controls during dog-human social interaction was significantly higher than that in the context without social interaction (Fig. 6C).

Pupil dilation synchrony was apparently affected by *Shank3*. Heterozygous −496 bp/+ and −483 + 7 bp/+ mutants (but not 1279 + 1 bp/+) showed no pupil dilation synchrony during dog-human interactions, and all three heterozygous mutants (−1279 + 1 bp/+, −483 + 7 bp/+ and −496 bp/+) showed a significantly lower pupil dilation synchrony index than WT control (Fig. 6C). As a control, there was no significant difference in pupil dilation synchrony index between WT and mutant dogs in the context without dog-human social interaction (Fig. 6C).

## DISCUSSION

Atypical sensory responses are part of the ASD diagnostic criteria [44]. Specifically, hyper- or hypo-reactivity responses to sensory inputs (e.g., apparent indifference to pain/temperature and altered responses to specific sounds or textures) are commonly observed symptoms in individuals with ASD [], which are also causes of significant distress and discomfort across the lifespan of people with autism. Previous studies suggested pupillary responses are reliable indicators for activity of the autonomic nervous system (ANS) [6, 55]. Abnormal ANS activity has been linked to a range of neurodevelopmental disorders, including ASD [56]. In the present

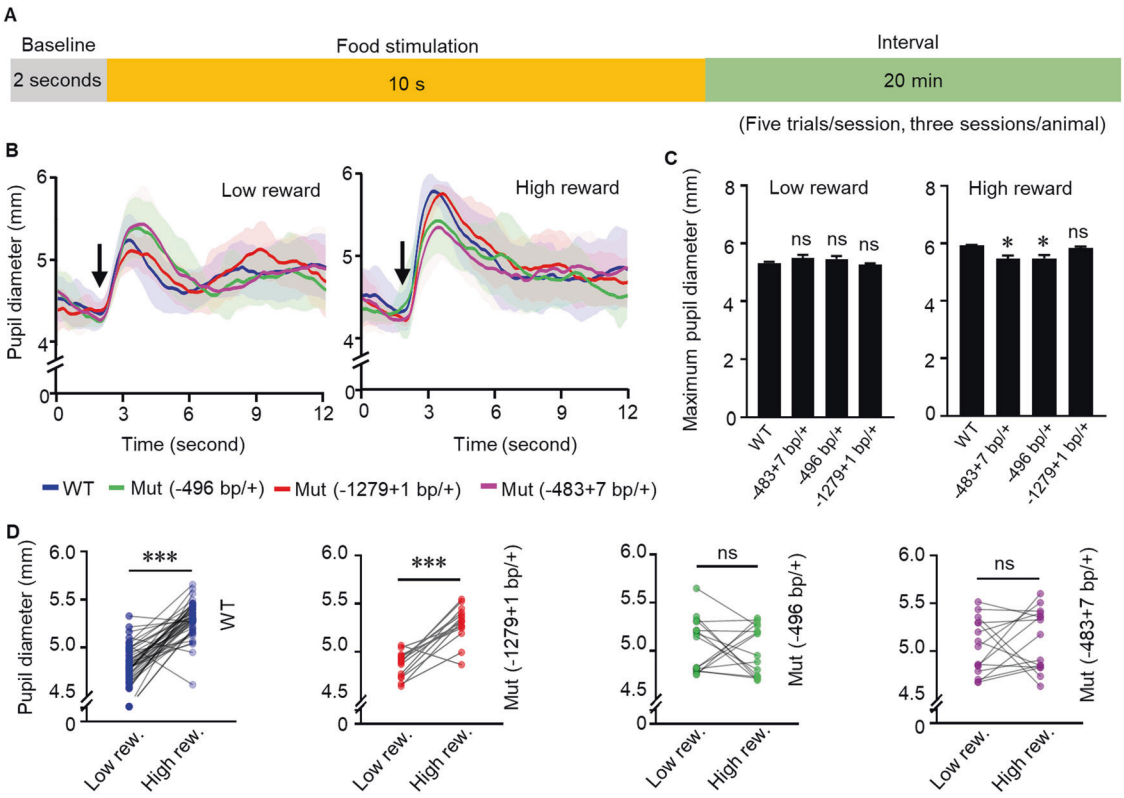


**Fig. 5 High-reward food is unable to induce a stronger pupil dilation response in *Shank3* mutant dogs. A** A food stimulus paradigm includes three blocks: grey = baseline recording of pupil diameter, orange = food stimulation, and green = inter-stimulus interval. **B** Pupil diameter as a function of time before and after low/high-reward food stimulation in WT and *Shank3* mutant dogs (color-coded). WT, wild-type; Mut, mutant. Black arrow indicates stimulus onset. 15 low-/15 high-reward food stimuli for each animal. **C** Maximum pupil diameter after low/high reward food stimulus in wild-type and *Shank3* mutant dogs. Error bars represent SEM. *$p < 0.05$; ns, no significance compared with WT controls (Kruskal-Wallis test). **D** Comparison of pupil diameter after low/high reward food stimulation in WT and *Shank3* mutant dogs. ***$p < 0.001$; ns, no significance (Wilcoxon test).

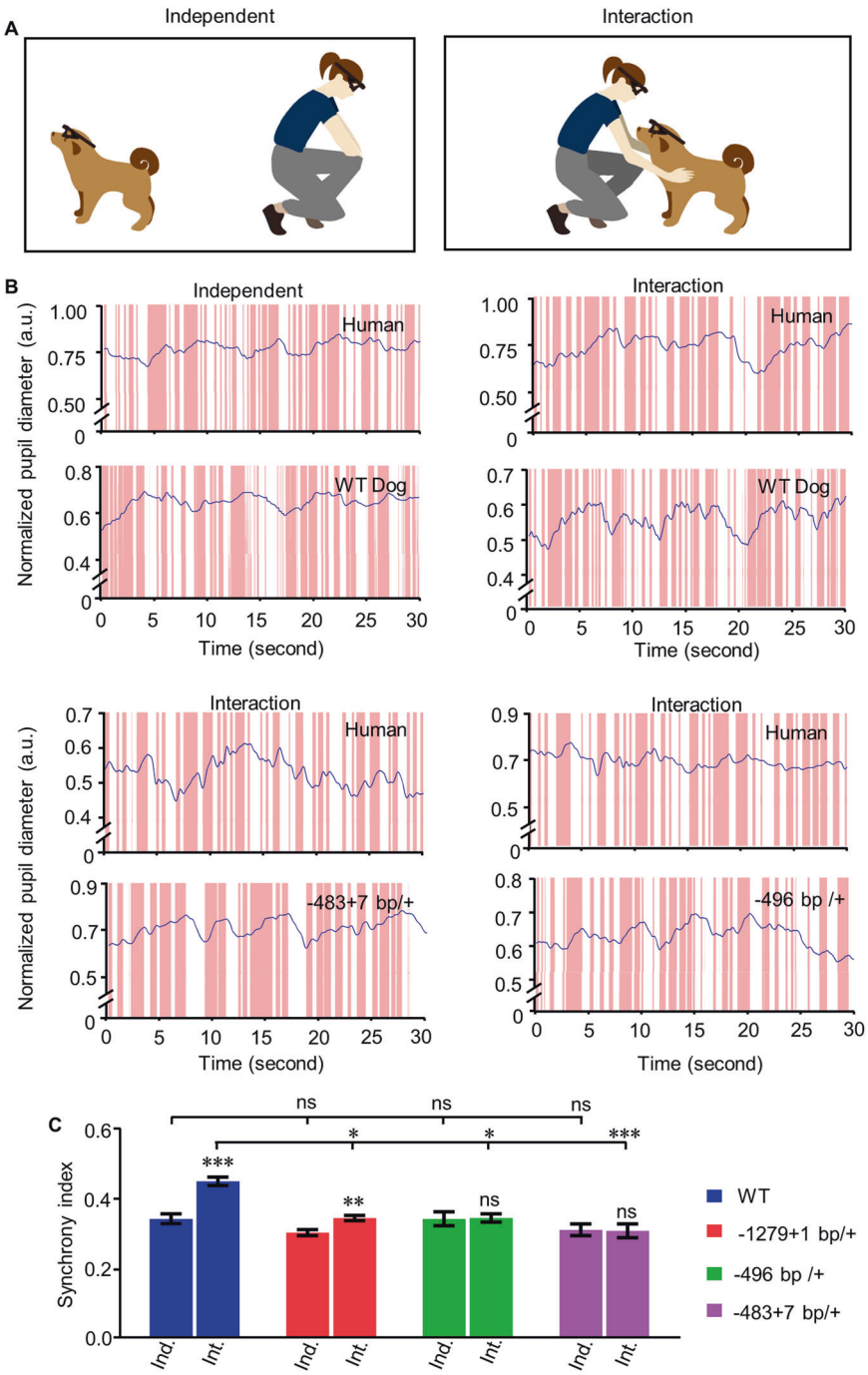


**Fig. 6 Loss of pupil dilation synchrony in *Shank3* mutants during dog-human interaction. A** The dog-human interaction paradigm includes two blocks: human and dog in the same room without mutual gaze and petting (left), and human interacting with the dog with mutual gaze and petting (right). **B** Examples of normalized pupil diameter as a function of time under independent and social interaction conditions in WT and *Shank3* mutant dogs. The pupil dilation process is highlighted in pink. **C** Pupil dilation synchrony index under independent (Ind.) and social interaction (Int.) conditions in WT and *Shank3* mutant dogs (color-coded). Error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significance (Mann-Whitley test for intra-group comparison and Kruskal-Wallis test for inter-group comparison). WT, wild type.

study, we developed head-mounted eye-tracker and deep learning-based pupil-detection algorithm. The altered pupillary responses induced by light, sound, food, and social interaction stimuli in mutant dogs, summarized in Fig. 7, are likely to be associated with atypical functioning of the ANS.

Balance between inhibitory and excitatory activity within the sympathetic and parasympathetic systems of the ANS determines the level of ANS activity. For light stimuli, we also observed a smaller constriction amplitude and longer pupillary light reflex latency in *Shank3* mutants, recapitulating that in autistic children [11, 12]. For the auditory stimuli, our findings in *Shank3* mutants are in agreement with a previous finding of increased pupil dilation to auditory stimulation in autistic patients [49, 57, 58] and hypersensitive responses to repeated sounds [13], suggesting similar auditory arousal (pupil dilation response) between dogs and humans. The altered pupillary responses in *Shank3* mutant dogs after the light/auditory stimulation may be explained by misregulation of the inhibitory activity of the parasympathetic

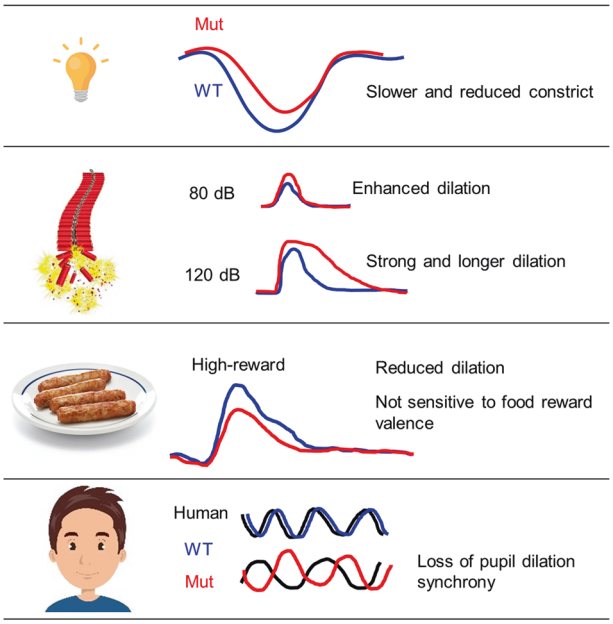


**Fig. 7 Overview of altered pupil responses in *Shank3* mutant dogs.** Pupil responses to non-social (light, sound, and food) and social (dog-human interaction) stimuli in WT (blue lines) and *Shank3* mutant (red lines) dogs.

system, the excitatory activity of sympathetic system of the ANS, or both.

Previous studies on pupil responses in humans and mice have revealed pupil dilation in two temporal phases, each with a specific underlying neural circuit. Rapid pupil dilation is closely associated with phasic activity of noradrenergic neurons, induced by activation of the sympathetic nervous system; by contrast, sustained pupil dilation during avoidance responses is accompanied by the activity of cholinergic axons, induced by inhibition of the parasympathetic nervous system [14, 59, 60]. The longer P1 and absence of P2 observed in *Shank3* mutants suggest they may have an altered ANS activity.

The different results of pupil responses to 80 dB and 120 dB stimuli may be explained by the Petersen and Posner model on attentional processing [61], which includes three systems of attention: the alerting system, the orienting system, and the executive system. The alerting system is responsible for maintaining a state of general arousal and readiness to respond to incoming stimuli; the orienting system directs attention towards a target stimulus, and allows individuals to focus on relevant stimuli while ignoring irrelevant or distracting information [61]. Previous studies in humans attribute the first phase (P1) of pupil dilation to the activation of the alert system and the second phase (P2) of dilation to activation of the orienting system [62]. Both 80 dB and 120 dB sound stimuli may activate the alerting system. However, unlike the mild 80 dB stimulus, the 120 dB stimulus may be considered a startling stimulus [63]. It is possible that dogs require activation of the orienting system for loud auditory stimuli to locate the source of the threat and perform avoidance movements. Thus, the P2 phase of pupil dilation induced by 120 dB stimuli may be controlled by orienting system. The loss of the P2 phase of the dilation in response to 120 dB stimuli in *Shank3* mutants indicates that the orienting system may be impaired, as suggested by a recent study on ASD children [64].

For food stimuli, food pictures have been used to induce pupil dilation in humans [65], but no studies on pupillary responses to actual food stimuli have been reported in both humans and dogs. Our work showed for the first time that food stimuli also induced pupil dilation in dogs. Interestingly, in comparison with control dogs, *Shank3* mutants showed reduced dilation to a high-reward food and did not react to a high-reward food with stronger pupil dilation, indicating hyper-activation of the parasympathetic system, hypoactivation of the sympathetic system, or both. Unlike auditory stimuli, which are received passively, food stimuli attract attention. Therefore, appropriate attention needs to be devoted to the food stimulus. Given that abnormal attention allocation has been reported in patients with ASD [66, 67], it is plausible that abnormal attention allocation may lead to neglect of or inability to pay full attention to high-reward food stimuli in *Shank3* mutant dogs.

For dog-human interactions, pupil dilation synchrony may be caused by coupled activity of the sympathetic and parasympathetic systems of the ANS. The shared attention between human and dog during social interaction may cause synchronized ANS activity, which in turn leads to pupil dilation synchronization. *Shank3* mutants showed no pupil dilation synchronization, indicating that ANS activity may not be synchronized during dog-human social interaction. It is possible that abnormal attention allocation during social interaction may cause atypical functioning of the ANS, which in turn leads to a loss of pupil dilation synchronization. Dysfunctional ANS due to abnormal attention allocation provides a possible mechanism for the widespread abnormalities in social interactions among individuals with autism.

We note the pupillometry for dogs we developed was robust, as the phenotypic variations in individual WT animals are minimum and only noticeable when stimulated by strong 120 dB sound stimuli. We observed consistently that the $-1279+1$ bp/+ mutant displayed a weaker pupillary response than the other two mutants. Specifically, during dog-human interaction, $-1279+1$ bp/+ mutant showed weaker pupil dilation synchrony compared with WT controls, while the two other mutants lost the pupil dilation synchrony. After light stimulation, mild auditory stimulation, and food stimulation, the mutant of $-1279+1$ bp/+ but not $-496$ bp/+ and $-483+7$ bp/+ mutants showed normal pupil response. The different phenotypes between three mutants may be because the $-1279+1$ bp/+ mutation is an in-frame deletion that results in a weak loss-of-function allele [29]. The similar phenotypes with different severity among three mutants further support that the altered pupillary responses to sensory stimuli are indeed caused by *Shank3* mutations.

In summary, we successfully designed and applied pupillometry to a dog model carrying different *Shank3* mutations and observed atypical sensory responses that are consistent with those reported in human autistic patients. We believe the methodology that we developed in the present study is useful for understanding the neural mechanisms underlying abnormal sensory experiences in ASD, and our findings provide potential biomarkers for diagnosis and drug development for autism. In future studies, the pupillometry can be combined with electrophysiological and neuroimaging assays in dogs to uncover the neural mechanisms underlying the atypical sensory responses in autistic, and perhaps other psychiatric patients.

## REFERENCES

1. Zokaei N, Board AG, Manohar SG, Nobre AC. Modulation of the pupillary response by the content of visual working memory. Proc Natl Acad Sci USA. 2019;116:22802–10.
2. Laeng B, Sirois S, Gredebäck G. Pupillometry: a window to the preconscious? Perspect Psychol Sci. 2012;7:18–27.
3. Bradley MM, Miccoli L, Escrig MA, Lang PJ. The pupil as a measure of emotional arousal and autonomic activation. Psychophysiology. 2008;45:602–7.
4. Graur S, Siegle G. Pupillary motility: bringing neuroscience to the psychiatry clinic of the future. Curr Neurol Neurosci Rep. 2013;13:365.
5. Martineau J, Hernandez N, Hiebel L, Roché L, Metzger A, Bonnet-Brilhault F. Can pupil size and pupil responses during visual scanning contribute to the diagnosis of autism spectrum disorder in children? J Psychiatr Res. 2011;45:1077–82.

6. de Vries L, Fouquaet I, Boets B, Naulaers G, Steyaert J. Autism spectrum disorder and pupillometry: a systematic review and meta-analysis. Neurosci Biobehav Rev. 2021;120:479–508.
7. Kleberg JL, Hanqvist C, Serlachius E, Högström J. Pupil dilation to emotional expressions in adolescent social anxiety disorder is related to treatment outcome. J Anxiety Disord. 2019;65:26–33.
8. Keil V, Hepach R, Vierrath S, Caffier D, Tuschen-Caffier B, Klein C, et al. Children with social anxiety disorder show blunted pupillary reactivity and altered eye contact processing in response to emotional faces: Insights from pupillometry and eye movements. J Anxiety Disord. 2018;58:61–9.
9. Silk JS, Dahl RE, Ryan ND, Forbes EE, Axelson DA, Birmaher B, et al. Pupillary reactivity to emotional information in child and adolescent depression: links to clinical and ecological measures. Am J Psychiatry. 2007;164:1873–80.
10. Sekaninova N, Ondrejka I, Bona Olexova L, Visnovcova Z, Mestanik M, Cesnekova D, et al. Oculometric behavior assessed by pupil response is altered in adolescent depression. Physiol Res. 2019;68:S325–38.
11. Fan X, Miles JH, Takahashi N, Yao G. Abnormal transient pupillary light reflex in individuals with autism spectrum disorders. J Autism Dev Disord. 2009;39:1499–508.
12. Daluwatte C, Miles JH, Christ SE, Beversdorf DQ, Takahashi TN, Yao G. Atypical pupillary light reflex and heart rate variability in children with autism spectrum disorder. J Autism Dev Disord. 2013;43:1910–25.
13. Zhao S, Liu Y, Wei K. Pupil-linked arousal response reveals aberrant attention regulation among children with autism spectrum disorder. J Neurosci. 2022;42:5427–37.
14. Reimer J, Froudarakis E, Cadwell CR, Yatsenko D, Denfield GH, Tolias AS. Pupil fluctuations track fast switching of cortical states during quiet wakefulness. Neuron. 2014;84:355–62.
15. Kuraoka K, Nakamura K. Facial temperature and pupil size as indicators of internal state in primates. Neurosci Res. 2022;175:25–37.
16. Artoni P, Piffer A, Vinci V, LeBlanc J, Nelson CA, Hensch TK, et al. Deep learning of spontaneous arousal fluctuations detects early cholinergic defects across neurodevelopmental mouse models and patients. Proc Natl Acad Sci USA. 2020;117:23298–303.
17. Zhou Y, Sharma J, Ke Q, Landman R, Yuan J, Chen H, et al. Atypical behaviour and connectivity in SHANK3-mutant macaques. Nature. 2019;570:326–31.
18. Zhao H, Jiang YH, Zhang YQ. Modeling autism in non-human primates: opportunities and challenges. Autism Res. 2018;11:686–94.
19. Jennings CG, Landman R, Zhou Y, Sharma J, Hyman J, Movshon JA, et al. Opportunities and challenges in modeling human brain disorders in transgenic primates. Nat Neurosci. 2016;19:1123–30.
20. Feng G, Jensen FE, Greely HT, Okano H, Treue S, Roberts AC, et al. Opportunities and limitations of genetically modified nonhuman primate models for neuroscience research. Proc Natl Acad Sci USA. 2020;117:24022–31.
21. Xiong Y, Hong H, Liu C, Zhang YQ. Social isolation and the brain: effects and mechanisms. Mol Psychiatr. 2023;28:191–201.
22. Wang G-D, Zhai W, Yang H-C, Fan R-X, Cao X, Zhong L, et al. The genomics of selection in dogs and the parallel evolution between dogs and humans. Nat Commun. 2013;4:1860.
23. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 2005;438:803–19.
24. Hong H, Zhao Z, Huang X, Guo C, Zhao H, Wang GD, et al. Comparative proteome and cis-regulatory element analysis reveals specific molecular pathways conserved in dog and human brains. Mol Cell Proteom. 2022;21:100261.
25. Pongrácz P, Rieger G, Vékony K. Grumpy dogs are smart learners-the association between dog-owner relationship and dogs' performance in a social learning task. Animals. 2021;11:961.
26. Törnqvist H, Somppi S, Koskela A, Krause CM, Vainio O, Kujala MV. Comparison of dogs and humans in visual scanning of social interaction. R Soc Open Sci. 2015;2:150341.
27. Koyasu H, Kikusui T, Takagi S, Nagasawa M. The gaze communications between dogs/cats and humans: recent research review and future directions. Front Psychol. 2020;11:613512.
28. Bunford N, Andics A, Kis A, Miklósi Á, Gácsi M. Canis familiaris as a model for noninvasive comparative neuroscience. Trends Neurosci. 2017;40:438–52.
29. Tian. R, Li. Y, Zhao. H, Zuo. Z, Zhao. J, Sun. Z, et al. Modeling SHANK3-associated autism spectrum disorder in beagle dogs via CRISPR/Cas9 gene editing (accepted).
30. Topál J, Román V, Turcsán B. The dog (*Canis familiaris*) as a translational model of autism: It is high time we move from promise to reality. Wiley Interdiscip Rev Cogn Sci. 2019;10:e1495.
31. Völter CJ, Huber L. Dogs' looking times and pupil dilation response reveal expectations about contact causality. Biol Lett. 2021;17:20210465.
32. Williams FJ, Mills DS, Guo K. Development of a head-mounted, eye-tracking system for dogs. J Neurosci Methods. 2011;194:259–65.
33. Jiang Y-H, Ehlers MD. Modeling autism by SHANK gene mutations in mice. Neuron. 2013;78:8–27.
34. Pelgrim MH, Espinosa J, Buchsbaum D. Head-mounted mobile eye-tracking in the domestic dog: A new method. Behav Res Methods. 2022;55:1924–41.
35. Bradski G. The openCV library. Dr Dobb's J: Softw Tools Professional Program. 2000;25:120–3.
36. Shelhamer E, Long J, Darrell T. Fully convolutional networks for semantic segmentation. IEEE Trans Pattern Anal Mach Intell. 2015; 3431–40.
37. Ronneberger O, Fischer P, Brox T. U-net: convolutional networks for biomedical image segmentation. Proc Int Conf Med Image Comput. Comput.-Assisted Intervention 2015; 234–41.
38. He K, Zhang X, Ren S, Sun J. Deep residual learning for image recognition. Proc. IEEE Conf Comput Vis Pattern Recognit. 2016; 770–78.
39. Szegedy C, Ioffe S, Vanhoucke V, Alemi A. Inception-v4, inception-resnet and the impact of residual connections on learning. Proc AAAI Conf Artif Intell. 2017;31:1.
40. Sandler M, Howard A, Zhu M, Zhmoginov A, Chen L-C. Mobilenetv2: Inverted residuals and linear bottlenecks. Proc IEEE Conf Comput Vis Pattern Recognit 2018; 4510–20.
41. Chollet F. Xception: Deep learning with depthwise separable convolutions. Proc IEEE Conf Comput Vis Pattern Recognit. 2017; 1251–8.
42. Mathis A, Mamidanna P, Cury KM, Abe T, Murthy VN, Mathis MW, et al. DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. Nat Neurosci. 2018;21:1281–9.
43. Dinalankara DMR, Miles JH, Nicole Takahashi T, Yao G. Atypical pupillary light reflex in 2-6-year-old children with autism spectrum disorders. Autism Res. 2017;10:829–38.
44. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. American Psychiatric Publishing: Washington, DC, 2013.
45. Williams ZJ, He JL, Cascio CJ, Woynaroski TG. A review of decreased sound tolerance in autism: Definitions, phenomenology, and potential mechanisms. Neurosci Biobehav Rev. 2021;121:1–17.
46. Landon J, Shepherd D, Lodhia V. A qualitative study of noise sensitivity in adults with autism spectrum disorder. Res Autism Spectr Disord. 2016;32:43–52.
47. Hayward SM, Mcvilly KR, Stokes MA. Autism and employment: What works. Res Autism Spectr Disord. 2019;60:48–58.
48. Shull-Selcer EA, Stagg W. Advances in the understanding and treatment of noise phobias. Vet Clin North Am Small Anim Pr. 1991;21:353–67.
49. Liao H-I, Kidani S, Yoneya M, Kashino M, Furukawa S. Correspondences among pupillary dilation response, subjective salience of sounds, and loudness. Psychon Bull Rev. 2016;23:412–25.
50. Cascio CJ, Foss-Feig JH, Heacock JL, Newsom CR, Cowan RL, Benningfield MM, et al. Response of neural reward regions to food cues in autism spectrum disorders. J Neurodev Disord. 2012;4:9.
51. Cascio CJ, Foss-Feig JH, Heacock J, Schauder KB, Loring WA, Rogers BP, et al. Affective neural response to restricted interests in autism spectrum disorders. J Child Psychol Psychiatry. 2014;55:162–71.
52. Kang O, Wheatley T. Pupil dilation patterns spontaneously synchronize across individuals during shared attention. J Exp Psychol Gen. 2017;146:569–76.
53. Cheng Y, Liu W, Yuan X, Jiang Y. The eyes have it: perception of social interaction unfolds through pupil dilation. Neurosci Bull. 2021;37:1595–8.
54. Wheatley T, Kang O, Parkinson C, Looser CE. From mind perception to mental connection: Synchrony as a mechanism for social understanding. Soc Personal Psychol Compass. 2012;6:589–606.
55. Hall CA, Chilcott RP. Eyeing up the future of the pupillary light reflex in neurodiagnostics. Diagnostics. 2018;8:19.
56. Hajcak G, Patrick CJ. Situating psychophysiological science within the Research Domain Criteria (RDoC) framework. Int J Psychophysiol. 2015;98:223–6.
57. Zekveld AA, Koelewijn T, Kramer SE. The pupil dilation response to auditory stimuli: current state of knowledge. Trends Hear. 2018;22:2331216518777174.
58. Rudling M, Nyström P, Bölte S, Falck-Ytter T. Larger pupil dilation to nonsocial sounds in infants with subsequent autism diagnosis. J Child Psychol Psychiatry. 2022;63:793–801.
59. Steinhauer SR, Siegle GJ, Condray R, Pless M. Sympathetic and parasympathetic innervation of pupillary dilation during sustained processing. Int J Psychophysiol. 2004;52:77–86.
60. Steinhauer SR, Hakerem G. The pupillary response in cognitive psychophysiology and schizophrenia. Ann N. Y Acad Sci. 1992;658:182–204.
61. Posner MI, Petersen SE. The attention system of the human brain. Annu Rev Neurosci. 1990;13:25–42.
62. Geva R, Zivan M, Warsha A, Olchik D. Alerting, orienting or executive attention networks: differential patters of pupil dilations. Front Behav Neurosci. 2013;7:145.

63. Dale AR, Walker JK, Farnworth MJ, Morrissey SV, Waran NK. A survey of owners' perceptions of fear of fireworks in a sample of dogs and cats in New Zealand. N. Zeal Vet J. 2010;58:286–91.
64. Ridderinkhof A, de Bruin EI, van den Driesschen S, Bögels SM. Attention in children with autism spectrum disorder and the effects of a mindfulness-based program. J Atten Disord. 2020;24:681–92.
65. Rebollo I, Schmidt M, Longren L, Park S. Influence of visual food cues on autonomic activity and craving. Biol Psychol. 2021;165:108197.
66. Murray D, Lesser M, Lawson W. Attention, monotropism and the diagnostic criteria for autism. Autism. 2005;9:139–56.
67. Goldstein G, Johnson CR, Minshew NJ. Attentional processes in autism. J Autism Dev Disord. 2001;31:433–40.

## ACKNOWLEDGEMENTS

We thank W. Zhou and Y. Jiang for discussion. This work was supported by grants from The National Key Research and Development Program (2019YFA0707100 and 2021ZD0203901 to Y.Q. Zhang), the Strategic Priority Research Program B of the Chinese Academy of Sciences (XDBS1020100 to Y.Q. Zhang), STI2030-Major Projects (2021ZD0203900 to P.F.Wei), the National Science Foundation of China (31830036 and 31921002 to Y.Q. Zhang, and 32222036 to P. Wei), and the Spring City plan (2022SCP001 to Y.Q. Zhang). The corresponding author (YQZ) had full access to all the data in the study and final responsibility for the decision to submit to publication.

## AUTHOR CONTRIBUTIONS

Y.Z., P.W., L.W., and K.G. conceptualized the project, supervised data collection and analyses. K.H., Q.Y., and W.R. collaborated in designing and constructing the apparatus. K.H. developed the software and algorithm for pupillometry. W.R. and Y.L. performed behavioral experiments, data collection, and analyses. W.R., Y.L. and Y.Z. wrote the manuscript.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41380-023-02277-8.

**Correspondence** and requests for materials should be addressed to Kun Guo, Pengfei Wei or Yong Q. Zhang.