TECHNICAL ABSTRACT:

The cerebellum plays a critical role in integrating sensory inputs to guide action. Autistic children and their siblings show atypical sensory responsiveness. The cerebellum is among the most-frequently disrupted brain regions in autistic patients. Furthermore, cerebellar injury associated with premature birth is followed by reduced prefrontal volume and an approximately 40-fold increase in ASD risk by age two – higher than the 10-20-fold risk associated with having a dizygotic ASD twin. Thus cerebellar processing is a candidate substrate for ASD integrative deficits, and might even play a role in driving the ontogeny of social deficits that are at the core of ASD.

We propose to apply in vivo optical imaging methods to test the hypothesis that cerebellar sensory representations and related forms of plasticity are disrupted in mouse models of autism. Optical methods open the possibility of monitoring up to hundreds of neurons, and when the probe is a genetically-encoded indicator such as GCaMP, the data include information on cell type identity. In autism model mice, we will image sensory representations under two conditions: suppression of stimulus representations by locomotion, and eyeblink conditioning, which requires cerebellar plasticity.

First, we will use somatosensory gating and eyeblink conditioning to probe disruptions in the function of the climbing fiber pathway, the teaching pathway to the cerebellum. In Purkinje cell dendrites, calcium transients reflect drive via the inferior olive/climbing fiber pathway. We will test whether complex spike population encoding is impaired in ASD model mice. In lobule VI we will quantify the encoding of corneal airpuff stimuli before and after delay eyeblink conditioning, a form of cerebellar learning. We will test the degree to which deficiencies in encoding can account for impairments in eyeblink conditioning.

Second, we will test for risk factors that may exacerbate a genetically driven cerebellar phenotype. We will also test whether social disruptions are correlated with deficits in cerebellar function. We have observed heterogeneity in delay eyeblink conditioning in *Shank3+/ΔC* and *Cntnap2-/-* mice. We will test the hypothesis that in these mice, cerebellar learning in the adult is susceptible to maternal separation stress, a manipulation that impairs eyeblink conditioning and deep nuclear dendritic spiking. We will also measure the degree to which cerebellar learning is correlated with social impairment.

Third, we will monitor function of cerebellar granule cell populations. Cerebellar granule cells are the most abundant neuron type in the brain, and represent a point of maximum divergence where stimuli can be represented by a highly distributed population code. Using recently developed Fast-GCaMP variants that respond with unprecedented speed to changes in calcium concentration, we will test the hypothesis that conditioned-stimulus representations are disrupted in autism models. Finally, we will attempt a rescue experiment to restore granule cell function and eyeblink conditioning.

These studies will identify how perturbation of genes associated with autism affect circuit-level cerebellar coding. In the long term, multiphoton optical imaging and eyeblink conditioning can be ported to other laboratories to provide a means of characterizing cerebellar function in mouse autism models.

Principal Investigator Name:	Principal Investigator Institution:	Principal Investigator Email:
Samuel S.-H. Wang	Princeton University	sswang@princeton.edu
Project Title		
Optical approaches to cerebellar circuit function in autism mouse models		

SPECIFIC AIMS. We propose to apply in vivo optical imaging methods to test the hypothesis that cerebellar sensory representations and related forms of plasticity are disrupted in mouse models of autism. We will image the brains of awake head-fixed mice, a preparation in which we have performed multiphoton microscopy using genetically encodable calcium indicators to examine neural correlates of sensory gating and associative learning. We will image sensory representations under two conditions: suppression of stimulus representations by locomotion, and eyeblink conditioning, which requires cerebellar plasticity. Our hypotheses fall along three Aims:

AIM 1: Are complex-spike representations of aversive stimuli perturbed in autism models? We will use somatosensory gating and eyeblink conditioning to probe disruptions in the function of cerebellum and related neural systems. In lobule IV/V, aversive somatosensory stimuli are encoded both by the probability of complex spike events in individual Purkinje cell dendrites, and by the synchronous activation of multiple dendrites. In Purkinje cell dendrites, calcium transients reflect drive via the inferior olive/climbing fiber pathway. We will test whether encoding is reduced in ASD model mice. In lobule VI we will quantify the encoding of corneal airpuff stimuli before and after delay eyeblink conditioning, the degree to which encoding is suppressed by a conditioned stimulus (auditory tone or light flash), and whether deficiencies in encoding can account for impairments in delay eyeblink conditioning.

AIM 2. Is variation in cerebellar function associated with postnatal stressors and social endophenotypes? Early-postnatal cerebellar disruptions are associated with autism, raising the possibility that cerebellar dysfunction can be used as a biomarker for aberrant developmental paths. We have observed heterogeneity in delay eyeblink conditioning in *Shank3+/ΔC* and *Cntnap2-/-* mice. We will test the hypothesis that in these mice, cerebellar learning in the adult is susceptible to maternal separation stress, which in wild-type rodents impairs eyeblink conditioning and deep nuclear dendritic spiking. We will also measure the degree to which cerebellar learning is correlated with social impairment.

AIM 3. Are granule cell representations of conditioned stimuli perturbed in autism models? Granule cells represent a point of maximum divergence in the mossy fiber pathway, and allow stimuli to be represented by a highly distributed population code. In granule cells, calcium transients reflect input via the mossy fiber pathway. Monitoring granule cell ensembles is technically challenging because of the close packing of the granule cell layer and because of the slow speed of older calcium indicator proteins. Using our recently developed Fast-GCaMP variants of GCaMP6f, we will test the hypothesis that conditioned-stimulus representations are disrupted in autism models in which the mossy fiber-granule cell pathway is genetically perturbed. Finally, we will attempt a rescue experiment to restore eyeblink conditioning.

These studies will identify how perturbation of genes associated with autism affect circuit-level cerebellar coding. In the long term, multiphoton optical imaging and eyeblink conditioning can be ported to other laboratories to provide a means of characterizing cerebellar function in mouse autism models.

BACKGROUND. The cerebellum plays a critical role in processing sensory information to guide action (Barlow, 2002; Timmann *et al.*, 2010). Representations are conditional on an animal's state, as manifest in phenomena such as sensory gating (Apps, 2000), which can be imaged in awake head-fixed mice (Ozden et al., 2012); and the suppression of neural responses to self-movement-dependent touch (Blakemore et al., 1998). The cerebellum also integrates and associates sensory inputs. One well-studied example is classical (a.k.a. delay) eyeblink conditioning (**Figure 1**), in which an initially neutral stimulus (*e.g.* a tone or a light flash) becomes associated with a strong, co-terminating stimulus (corneal airpuff) that, by itself, evokes an unconditioned blink response. These functions are of relevance because autistic children and their siblings show atypical sensory responsiveness, including abnormalities in eyeblink conditioning (Sears *et al.*, 1994; Tobia and Woodruff-Pak, 2009). Thus cerebellar processing is a candidate substrate for ASD integrative deficits.

The cerebellum may also play cognitive and affective roles. In human adults, lesions of the posterior vermis lead to a cognitive-affective syndrome that includes disjointed thought, and in children, such damage causes language regression (Schmahmann, 2004; Timmann *et al.*, 2010). Sensory deficits in early life might developmentally impair the maturation of forebrain circuitry (Steinlin, 2008): cerebellar injury associated with premature birth is followed by reduced prefrontal volume and an approximately 40-fold increase in ASD risk by age two (Limperopoulos *et al.*, 2012) – higher than the 10-20-fold risk associated with having a dizygotic ASD twin. In mice, two studies in validated ASD models have shown that genetically-induced disruption of cerebellar Purkinje cells leads to autism-associated symptoms (Tsai *et al.*, 2012; Baudouin *et al.*, 2012). Thus the cerebellum might help guide the development of social and affective capacities.

The circuitry of the cerebellar cortex and nuclei follows general, repeating motifs (Apps and Garwicz, 2005), suggesting that insights into circuit function from one part of cerebellum might be generally informative about cerebellar function at large. In humans, default-mode imaging methods have revealed an overall organization in which the cerebellum projects to nearly the entire neocortex (Buckner et al., 2011). In animal experiments, rabies and herpesvirus

tracing have identified specific cerebellar connections with medial prefrontal cortex (Strick *et al.,* 2009), anterior cingulate cortex (Argüello *et al.,* 2012), and ventral tegmental area (Phillipson, 1979; Geisler and Zahm, 2005; Watabe-Uchida *et al.,* 2012). Thus a head-fixed mouse system for observing cerebellar circuitry might eventually shed light on a variety of whole-brain systems-level functions.

PRELIMINARY RESULTS. In vivo imaging of neural activity. Imaging methods add a new dimension to the study of neural coding. Traditionally, in vivo recording has used one or multiple electrodes to sample a subset of neurons in a volume. Optical methods open the possibility of monitoring up to hundreds of neurons, and when the probe is a genetically-encoded indicator such as GCaMP, the data include information on cell type identity. In recent years, calcium indicator proteins have shown greatly improved signal-to-noise properties. We have applied these methods to examine Purkinje cell dendritic calcium signals, which reflect complex spike activity, in sensory gating (Ozden et al., 2012) and in eyeblink conditioning (**Figure 2**).

A remaining challenge is to improve temporal resolution. We have developed Fast-GCaMPs, which have up to 20-fold faster response kinetics than existing GCaMPs (Sun et al., 2013). We have inserted our Fast-GCaMP mutations into a new Janelia Farm probe, GCaMP6f. The result, Fast-GCaMP6f-RS09, has equal brightness and substantially faster kinetics (**Figure 3, left**). Fast-GCaMPs will speed GC responses, which using existing technology (Ozden et al., 2012) decline with half-maximal times of <200 ms GCaMP6f (**Figure 3, right**).

Eyeblink conditioning. In collaboration with the Medina laboratory at U. Penn. (see letter of support), in head-fixed mice we have established eyeblink conditioning with acquisition, extinction, and timing properties similar to rabbit. Our treadmill-based method allows reliable screening of mouse delay eyeblink conditioning (Chettih *et al.,* 2011; Giovannucci *et al.,* 2011), a form of associative learning that requires the cerebellum (McCormick and Thompson, 1984).

Eyeblink conditioned stimulus (CS) and unconditioned stimulus (US) information streams converge on vermis lobule VI and hemispheric lobule VI (HVI, also known as simplex lobule) and interpositus (IP) nucleus, the known substrates for forming CS-US associations (**Figure 1**). US information reaches the cerebellum through a polysynaptic pathway that involves the sensory trigeminal nucleus and inferior olive (IO), whose CFs reach PCs in the cerebellar cortex and deep nuclear neurons via axon collaterals. The deep nuclei and cerebellar cortex are thought to play complementary roles in eyeblink conditioning. Anterior IP is essential for both learning and retention of the conditioned eyeblink response. The cerebellar cortex, especially lobules VI/HVI, is involved in both response acquisition and in making precise discriminations in learning parameters such as CS-US interval and the timing of the conditioned blink. This framework allows specific tests to probe the mechanism for the circuit defects we have seen so far.

We have found deficits in CR acquisition in multiple autism models: (1) the *Shank3ΔC* mutation associated with Phelan-McDermid syndrome (22q13.3 deletion syndrome) and (2) the knockout (-/-) of the mouse ortholog of *CNTNAP2* (Peñagarikano *et al.,* 2011). These studies have been presented at the SFN annual meeting (Kloth *et al.,* 2012). In addition, we have found different degrees of impairment in different models. We have measured CR acquisition deficits in *Dup(15q11-13)* mice (Nakatani et al., 2009) and poorly timed CRs in $MeCP2^{308}$ mice (Shahbazian et al., 2002; Ben-Shachar et al., 2009). These results demonstrate the applicability of delay eyeblink conditioning in a wide variety of mouse ASD models. In preliminary studies, we have observed inter-individual variation in *Shank3+/ΔC* and *Cntnap2-/-* mice (**Figure 4**). This increased variability is consistent with the idea that many risk alleles are of small effect, and may render a developing nervous system vulnerable to environmental insults or additional genetic risks.

SIGNIFICANCE OF THE PROPOSED STUDIES. Current work in mouse autism models does not include characterization of cerebellar function as a standard practice. The closest assay in common use is rotorod, a general measure of motor coordination. Our use of eyeblink conditioning takes advantage of over 30 years of characterization, both of classical conditioning as well as trace conditioning (which recruits multiple brain regions working in concert, including the cerebellum; Weiss and Disterhoft, 2011; Siegel et al., 2012).

Many tasks involving cerebellum require the integration of information streams from different senses. Eyeblink conditioning usually pairs a tone (auditory) or light (visual) flash with a corneal airpuff (touch), and vestibuloocular reflex gain adaptation relies on a vestibular-driven retinal slip signal (visual-vestibular mismatch). Monitoring cerebellar sensory function in a head-fixed mouse is straightforward because sensory gating requires only locomotion, and eyeblink conditioning is fast and robust when the mouse is allowed to walk on a treadmill. In the long term, eyeblink conditioning can be extended to a trace paradigm (**Figure 5**; Siegel et al., 2012), opening the possibility of characterizing the interplay of cerebellum with other major brain divisions including hippocampus and neocortex. Head-fixed methods also lend themselves well to virtual-reality approaches now in use at Princeton (Domnisoru et al., 2013).

Nonoverlap with a Nancy Lurie Marks Family Foundation (NLMFF) project. The NLMFF has made a grant to Mustafa Sahin (Boston Children's Hospital / Harvard Medical School), Wade Regehr (Department of Neurobiology, Harvard Medical School), and myself to test for a developmental role for cerebellum in ASD. That proposal builds on the observation that behavioral deficits in cerebellar-Tsc1-knockout mice can be rescued by early postnatal administration of

rapamycin to compensate for the loss of Tsc, a negative regulator of mTOR (Tsai *et al.,* 2012). The NLMFF project specifically concerns a developmental model in which we are testing the hypothesis that early-life cerebellar dysfunction sets up ASD endophenotypes. In contrast, this SFARI proposal seeks to demonstrate cerebellar dysfunction in adult mice and test for gene-environment interactions. There is no conceptual or experimental overlap with the NLMFF project.

EXPERIMENTAL DESIGN

AIM 1: ARE REPRESENTATIONS OF AVERSIVE STIMULI PERTURBED IN MOUSE AUTISM MODELS?

In models of cerebellar learning, information storage can occur via plasticity at parallel fiber-Purkinje cell synapses, where long-term depression (LTD) is instructed by postsynaptic depolarization. Postsynaptic depolarization (in the form of complex calcium spikes) is driven by climbing fiber firing singly or in bursts (Mathy et al., 2009), or by dense activation of parallel fibers impinging on a part of the dendritic arbor (Eilers et al., 1995; Wang et al., 2000). These calcium signals can report the degree of dendritic depolarization and are necessary for inducing plasticity (Linden, 1999).

We will measure the representations of two kinds of aversive unconditional-stimulus (US) event, both of which are gated by expectations. In the following experiments, a US can be delivered to a naïve animal (sensory gating experiments) or after learning (eyeblink conditioning experiments), a condition that allows CS-US pairings to be tested.

In naïve animals, aversive events such as clap sounds and airpuff to the animal's hindflank trigger PC-dendritic and granule-cell responses that encode stimulus intensity and are strongly gated by locomotion (Ozden et al., 2012). In eyeblink conditioning, we will work with mice that have been trained to generate conditioned responses (CRs). Under this condition we can give either an unexpected US (i.e. US alone) or a predicted US (as in CS+US pairings). We have found that when USs are predicted, their representation in PC dendrites is suppressed (**Figure 2**). In ASD models with impaired acquisition of CRs, we will test whether US encoding is impaired. In PC dendritic calcium signals, we will measure (a) US-evoked repsonse probabilities, (b) US-evoked dendritic signal amplitudes, (c) co-activation across bands of dendrites, and (d) suppression of these three signals during CS+US pairing in a trained mouse.

1a. Is the encoding of instructive signals impaired at the level of individual PC dendrites? We will measure the amplitude and probability of dendritic calcium transients. In wild-type mice, we have found using GCaMP6f that dendritic signal amplitude can vary with corneal airpuff intensity (**Figure 6**), implying the novel finding that PC dendritic depolarization has analog features. The high brightness of GCaMP6f will allow us to observe calcium transients that fill part of the dendritic arbor, an event that can drive local plasticity (Wang et al., 2000).

Different ASD mouse models may have defects in distinct components of cerebellum circuitry. Shank3 is expressed in mossy fibers and granule cells (Boeckers *et al.,* 2001), and Cntnap2 is expressed in Purkinje cells (Paul *et al.,* 2012). Thus in initial characterization we will seek to find defects in mossy fiber representations of conditioned stimuli (CSs) (Kalmbach *et al.,* 2010) in Shank3 animals, and defects in unconditioned-stimulus representations in *Cntnap2-/-* mice. At the same time, any search for circuit mechanisms must be broad since acquisition of CRs requires both mossy fiber and climbing fiber pathways in the cerebellar cortex and the deep cerebellar nuclei, and furthermore may be modulated by amygdala, hippocampus, and neocortex (Lee and Kim, 2004; Strick *et al.,* 2009; Boele *et al.,* 2010).

The cellular patterns of expression suggest diverse outcomes for which we will be alert. For example, Cntnap2 participates in establishing GABAergic neurons throughout the brain (Peñagarikano et al., 2011). These neurons include Purkinje cells and some cerebellar interneurons (Paul et al., 2012), suggesting the possibility that PC dendritic signaling will be impaired. In preliminary anatomical measurements, we find that the molecular layer of *Cntnap2-/-* mice is thinner compared with *Cntnap2+/-* or wild-type littermates. In this case, altered calcium signaling may be manifest in the form of differences in subdendritic signaling. The probability of dendritic spikes may also be affected because Purkinje cells inhibit deep nuclear neurons which then feedback to inhibit the inferior olive, where the US is initially represented.

1b. Is sensory-evoked climbing fiber/complex spike synchrony impaired in ASD model mice? A second mechanism of US encoding is complex spike synchrony. Ensembles of PCs can fire complex spikes together with millisecond-level synchrony due to electrical coupling between olivary neurons (Ozden et al., 2009). We have previously found (Ozden et al., 2009, Ozden et al., 2012) that complex spike co-activation contains information that enhances the ability of ensembles of PC dendrites to encode stimulus intensity. Such co-activation may be read out by neurons in the deep cerebellar nuclei, whose dendrites receive olivonuclear excitation via climbing fiber collaterals and are capable of generating calcium-based regenerative signals (Schneider et al., 2013). Thus downstream convergence onto the interpositus provides a site where disruption of synchrony could have long-distance effects on the rest of the brain.

1c. Do impairments in unconditioned-stimulus representations account for eyeblink conditioning deficits?

Perturbations to Purkinje cell function would be predicted to affect response probability and response amplitude without affecting the specific time course of an individual CR. We will test these predictions at the level of individual mice in three ways: (1) We will use a robust, single-parameter measure of eyeblink conditioning, the fraction of CRs that exceed 15% of the amplitude of an unconditioned response (UR). (2) We will measure the response probability and response amplitude separately. (3) We will characterize the time course of the resulting learned CRs. We already have preliminary results for (1) and (3) that are consistent with our predictions. We have successfully applied the analysis required for (2) to

wild-type mice (**Figure 4**). We will start with *Cntnap2-/-* mice and then move on to *Shank3+/ΔC* mice, both of which show high phenotypic variability for eyeblink.

Pitfalls and alternatives. In addition to Purkinje cell dendritic signals, upstream mechanisms may also gate the salience of sensory events. To test for alterations at the extracerebellar level, we will measure auditory-auditory/visual-auditory pre-pulse inhibition and the unconditioned eyelid response to test whether the responses to conditioned stimuli and unconditioned stimuli, respectively, are intact. In preliminary results, there is no difference in the unconditioned response in *Shank3(+/ΔC)* and *Cntnap2-/-* versus wild-type; pre-pulse inhibition experiments also indicate no differences in prepulse startle to auditory stimuli.

If US representations are not altered, other steps in the induction of cerebellar plasticity may be impaired. As a followup to imaging experiments, we will test the properties of PF-PC plasticity. We will perform brain slice experiments to test the ability of PF+CF pairing to trigger synaptic plasticity. PF-PC synaptic plasticity shows bidirectionality (Jörntell and Hansel, 2006) and timing-dependence (Safo and Regehr, 2008), and relying on both mGluR-dependent (Wang *et al.*, 2000; Steinberg *et al.*, 2006) and mGluR-LTD is mGluR-independent mechanisms depending on the PF stimulus pattern and intensity (Wang *et al.*, 2000). We will first focus on mGluR-dependent LTD since this is both central to models of cerebellar learning, and because mGluR mechanisms have been implicated in other ASD models (for example see Auerbach *et al.*, 2011). Control experiments will be done to test whether changes in plasticity are mGluR-dependent. A second target of analysis is nonassociative PF-PC LTP, which is induced by 1 Hz PF-alone stimulation.

AIM 2. ASSOCIATION OF VARIABLE CEREBELLAR FUNCTION WITH POSTNATAL STRESS AND SOCIAL ENDOPHENOTYPES. Cerebellar disruptions are often found in persons with autism (Palmen et al., 2004; Courchesne et al., 2005; Wegiel et al., 2013)**,** and early-life cerebellar damage is associated with an approximately 30-fold increase in the later risk of ASD (Limperopoulos et al., 2012). These findings raise the possibility that cerebellar dysfunction is closely related in some way with the core deficits of autism. The causal nature of this relationship is an outstanding question. In one scenario, cerebellar damage might be developmentally "upstream" and influence the maturation of circuitry subserving social functions. In another, correlative scenario, disruptions leading to cerebellar dysfunction and to ASD's core deficits might have a common cause, such as combinations of genetic predisposing factors. In either case, it is relevant to ask whether cerebellum-specific dysfunction might be predictive of ASD-related endophenotypes.

2a. Is variability in cerebellar circuit function correlated with individual variation in social endophenotypes? We will test this using the variability in eyeblink-conditioning phenotypes that we have observed in Shank3 and Cntnap2 mice. Our fundamental framework is to test the hypothesis that the ability to produce learned eyeblink-CRs is correlated with degree of impairment in behavioral assays for ASD-like phenotypes. The tests to be performed are all in use in the laboratory of our collaborator Mustafa Sahin (Tsai, Hull et al., 2012; see letter of support).

We will test whether, on an animal-by-animal basis, behavioral phenotypes are correlated with impairments in eyeblink learning. Social interaction will be tested using a three-chambered assay of social approach, with time spent in the center chamber, in interaction with a novel mouse, or with a novel object as the metric. A social novelty paradigm will also be tested in which time spent with a novel vs. familiar animal is tested. Social interaction will also be tested using social vs. nonsocial olfactory cues. Cognitive inflexibility will be modeled using a reversal learning paradigm using a water T-maze. In this experiment the metric of inflexibility is the time taken to learn when an escape platform has been moved from one location to a reversed location. Finally, communication deficits will be measured by quantifying ultrasonic vocalization as a measure of abnormal mother-pup communication.

These experiments can potentially be accelerated depending on the results of Aims 1 and 2. If If in vivo imaging reveals a strong circuit-level phenotype in the sensory-gating task or in responses to US-alone corneal airpuffs, it will be possible to quantify cerebellar dysfunction in a single day of imaging. This would replace eyeblink conditioning, a protocol that typically takes 1-2 weeks to complete.

2b. Gene-environment interactions in cerebellar learning impairment. We will test whether the ability to acquire CRs is affected by environmental factors. Even after saturation of training, variation in CR production is greater in *Shank3+/ΔC* mice and *Cntnap2-/-* mice than littermate wild-type controls. Such variation, which occurs against a shared C57/BL6 genetic background, suggests the possibility that these transgenic mice are more susceptible to environmental influences than wild-type mice.

In mice, early-life stress affects cerebellar function at both the behavioral and cellular level. Neonatal maternal separation alters glucocorticoid receptor expression in the interpositus nucleus (Wilber and Wellman 2009), and leads to deficits in eyeblink conditioning that persist in adulthood (Wilber et al., 2011). The experience of being recently shipped increases corticosterone in both a dam and her pups, a change that is accompanied by a transient loss of calcium-based dendritic excitability in the deep cerebellar nuclei. Effects in the deep cerebellar nuclei persist for days (Schneider et al., 2013). These lines of evidence indicate that stress hormones can have a lasting impact on cerebellar maturation.

We will test whether stress-induced cerebellar disruption correlates with social dysfunction, and whether eyeblink and social phenotypes are influenced by maternal separation, a strressor that is known to disrupt cerebellar function. We will test whether this stressor can influence phenotypic outcomes in *Shank3* and *Cntnap2* models. We will perform maternal separation (Wilber et al., 2011) in which pups from the same litter are randomly assigned to either standard animal facilities rearing (control group) or a maternal separation group (1 h per day, postnatal days 2-14). This manipulation will be validated in pilot experiments to measure corticosterone levels (Schneider et al., 2013). Another litter of mice will be subjected to the same treatment, and tested as young adults (P60-90) for delay-eyeblink acquisition and social-function assays. We will test whether delay eyeblink conditioning is more strongly impaired in the ASD models than in wild-type mice, and whether eyeblink-conditioning and social phenotypes are correlated.

Pitfalls and alternatives. Eyeblink conditioning can be affected by forebrain-cerebellar interactions. Learning across trials and sessions is modulated by extracerebellar areas including amygdala, hippocampus, and neocortex (Lee and Kim, 2004; Strick et al., 2009; Boele et al., 2010). Hippocampal activity slows the time course of learning, while amygdalar input speeds learning (Lee and Kim, 2004), making eyeblink conditioning a possible bioassay for cerebellum-hippocampus and cerebellum-amygdala interactions. The prolonged timecourse of learning in *Cntnap2(-/-)* and *Shank3(+/ΔC)* mice might be caused by disruptions of interactions with forebrain regions. We will lesion the hippocampus and amygdala to test this hypothesis. Using electrolytic lesions, we will ascertain whether the resulting speeding and slowing of CR acquisition (Lee and Kim, 2004) are different from wild-type littermates. For example, if hippocampal deficit contributes to the delayed timecourse of learning, then a lesion of the dorsal hippocampus (Lee and Kim, 2004) should speed learning in wild-type mice but not in transgenic mice. Generally, if the performance of lesioned animals does not differ from that of unlesioned animals of the same strain, this would suggest that genetic alterations affected interactions between cerebellum and the site of lesion.

It should be noted that the Bangash et al., 2011 paper demonstrating ASD-like deficits in Shank3(+/ΔC) mice has been retracted due to problems not having to do with the behavioral data. In the event that difficulties do arise with the behavioral data, we have been offered access to another Shank3 model by J. Buxbaum (Mount Sinai School of Medicine).

AIM 3. ARE GRANULE CELL REPRESENTATIONS OF CONDITIONED STIMULI PERTURBED IN MOUSE AUTISM MODELS? Pathways encoding CS information arrive in the cerebellum via intermediate nuclei (e.g. auditory thalamic nuclei for tone information) and the pons, which gives rise to the mossy fiber (MF) projection. MFs terminate in IP and on granule cells (GCs), whose parallel fibers (PFs) excite Purkinje neurons and molecular layer interneurons. In mice expressing Shank3ΔC, which is made at high levels in cerebellar granule cells, conditioned blinks are too brief in their time course. This defect is consistent with a dysfunction in the representation of CS information by granule cells in cerebellar cortex. We will therefore perform GC imaging starting with *Shank3+/ΔC* mice.

3a. Is population encoding of conditioned stimuli impaired in granule cells? To evaluate stimulus encoding in the MF pathway, we will use calcium imaging to quantify the time course and pattern of GC activation in response to CS and US events. Granule cell activity is challenging to quantify using imaging methods for two reasons: (a) close packing of GCs generates a refractively heterogeneous environment, leading to poorer depth penetration compared with neocortex. (b) Calcium removal mechanisms from GCs are slow relative to the bandwidth of spiking activity, which can reach >300 Hz (Chadderton et al., 2004). To address (a), we will use recent versions of GCaMP with higher per-molecule brightness: GCaMP8 from Junichi Nakai's group (Ohkura et al., 2012), and GCaMP6f from the Janelia Farm Research Campus of HHMI, both of which have a maximal brightness of approximately double that of GCaMP3, which we have used before to image up to 100 GCs at once (Ozden et al., 2012). To address (b), we will use Fast-GCaMP mutations (**Figure 3**) and apply deconvolution methods (Vogelstein et al., 2010) to extract neural signaling events from in vivo signals. GC off-responses to calcium fall with $t_{1/2}$ values of 0.1-0.2 s, opening the possibility of resolving closely spaced firing events. Signals will be calibrated by comparison with brain slice recordings to image spike-related and EPSP-related signals.

Pitfalls and alternatives. Stimulus encoding may be disrupted at extracerebellar sites. We will vary the intensity of the conditioned stimulus (tone, whisker) to test if this can compensate for lost function and/or recruit additional extracerebellar learning mechanisms. If the defect results from extracerebellar influence, learning differences between *Shank3(+/ΔC)* and *Shank3(+/+)* may be specific to particular sensory modalities. If the defect is mossy-fiber specific, these forms of learning will be similar between the two genotypes for a variety of CSs. A necessary comparison in these experiments is to measure sensitivity at prior stages of processing with startle response testing. A second test is to measure reacquisition after extinction, a learning phenomenon that is thought to rely on deep nuclear learning mechanisms. Intact reacquisition would suggest that plasticity in the deep nuclei is functioning normally. Preliminary evidence suggests that this is the case, indicating that we have localized Shank3-related dysfunction to the cerebellar cortex.

3b. Do impairments in conditioned-stimulus representations account for conditioned-response deficits? The ΔC mutation of Shank3 is thought to negatively influence glutamatergic signaling (Bangash et al., 2011), and a related mutation leads to widespread reductions in synaptic transmission and altered cell morphology in the cerebellar cortex (Giza et al., 2010). We hypothesize that the performance and timing effects in our experiments are the result of deficits in

the mossy fiber pathway. We will test this idea in two ways: (1) by correlating encoding performance with eyeblink conditioning on an animal-by-animal basis, the same strategy as in Aim 1c; and (2) by using brain slice physiology.

In brain slices, we will perform three tests in Shank3(+/ΔC) and wild-type littermates: (1) Use whole-cell patch-clamp recording to test whether mossy fiber-granule cell synaptic transmission is perturbed, as quantified by failure rate and AMPA/NMDA ratio (Giza et al., 2010). (2) Measure paired-pulse facilitation of PF-PC synaptic transmission (Dittman et al., 2000), using climbing-fiber-Purkinje cell paired-pulse depression, which we predict will be unaffected, for comparison. (3) Measure the input-output (f-I) relationship of PCs, and (4) measure the amplitude of mGluR-dependent parallel fiber-Purkinje cell LTD from pairing PF and climbing fiber (CF) activity (Wang et al., 2000). We predict that experiments (1) and (2) will show perturbations.

Pitfalls and alternatives. GCs of interest may be located deep in the cerebellar cortex, where they will be difficult to image because of the refractive properties of the GC layer. If necessary, as a second approach to monitoring mossy fiber activity, we will image molecular layer interneurons, which are technically straightforward to visualize because of their sparse distribution (Ozden et al., 2012). These neurons' calcium signals scale up monotonically with firing rate and can be used as a general proxy for overall mossy fiber pathway activity.

3c. Can impairments in eyeblink conditioning be rescued by manipulation of mossy fiber signals? It has previously been demonstrated that direct pontine (i.e. mossy fiber) activation can take the place of a CS in driving eyeblink conditioning (Steinmetz et al., 1989; Freeman and Rabinak, 2004). We will test whether the genetically-induced disruptions in eyeblink conditioning we have observed can be rescued. We will evaluate three approaches in the following order: (1) cannulation followed by local infusion of allosteric enhancers of AMPA receptor activity, such as cyclothiazide and PEPA (Sekiguchi et al.,), which have been used in vivo to enhance excitatory transmission; (2) constitutive increases in GC activity using AAV-driven expression of the engineered G-protein coupled receptor hM3Dq (Alexander et al., 2009), which is activated by the exogenously applied drug clozapine-N-oxide (CNO); (3) Optogenetic activation of GCs using ChR2 during training sessions, in collaboration with I. Witten (Princeton). hM3Dq and ChR2 will be expressed using the same strategy that we use for GCaMP6f expression, packaging in adeno-associated virus (AAV) using the hSyn or GABAAalpha6 promoter.

Pitfalls and alternatives. Because circuitry underlying delay eyeblink conditioning is distributed across HVI and simplex lobule, drug or AAV injections will have to span a wide spatial range. AAV titers will be adjusted to allow spatially widespread expression. The range of spatial expression can be increased by preinjecting mice with mannitol to improve access in intercellular spaces (Kuhn et al., 2012).

NOVELTY. Our laboratory is among the few to optically monitor the neural correlates of cerebellar learning. Recent innovations in rodent head-fixed recording allow a circuit-level perspective on the neural basis of unexpected-event representation in the brain. Our advanced tools for in vivo imaging were developed during an ARRA grant for head-fixed and virtual reality-based methods for in vivo rodent recording, with David Tank as PI (Sam Wang and Carlos Brody, co-PIs). These steps are significant, since existing ASD mouse behavioral assays can often be challenging to merge with single-cell recording. In the future, the fact that mice perform well on a freely rotating treadmill opens the way to behavioral assays that include social olfactory cues, which are easily administered to a head-fixed mouse.

RELEVANCE TO AUTISM. The studies proposed here are relevant to autism in three ways. First, disrupted cerebellar anatomy and function are widespread in autistic persons, and some of these functions have been replicated in animal models such as Fragile X and tuberous sclerosis. Until now, these dysfunctions have not been examined at the microcircuit level. The studies described here open the possibility of characterizing circuit dysfunction in detail using modern molecular and optical methods. Second, the work has the potential to establish a cerebellum-based approach for understanding gene-environment interactions in early postnatal development. Third, these experiments will provide cerebellum-based biomarkers for ASD-associated circuit dysfunction and environmental susceptibility. In the long term, cerebellar function in mice may be used as an animal-based assay for the effectiveness of therapeutic treatments.

In addition, our ability to measure a circuit-level phenotype quantitatively may be useful in understanding the effect of combinations of genes of small effect. Why does the presence of a small-effect ASD susceptibility allele usually lead to a neurotypical result? Many given gene variants are likely to have differential effects on various components of brain circuitry. If core symptoms of ASD are caused by specific combinations of defects, it will be essential to understand what these combinations do individually and in combination.

TIMELINE AND MILESTONES. **Year 1:** *Q1-4*, Complete Aim 1. *Q3-4*, Begin social assays for Aim 2 for correlation with eyeblink conditioning. *Q3-4*, Optimize Fast-GCaMP6f-RS09 expression in granule cells for Aim 3. **Year 2:** *Q1-4*, Complete Aim 2 postnatal stress and social-assay experiments. *Q1-2*, Complete Aim 3.

Principal Investigator Name:	Principal Investigator Institution:	Principal Investigator Email:
Samuel S.-H. Wang	Princeton University	sswang@princeton.edu
Project Title Optical approaches to cerebellar circuit function in autism mouse models		

Figures

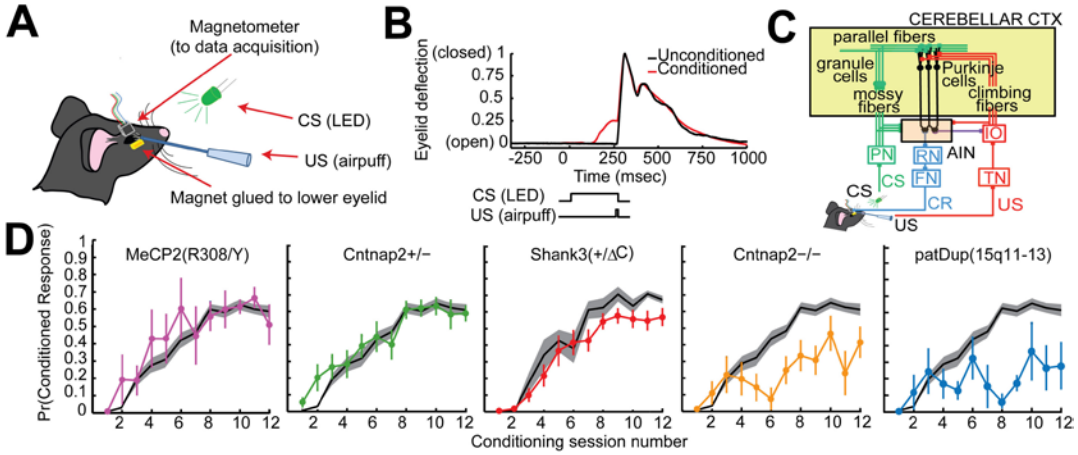

Figure 1. Eyeblink conditioning in mouse autism models. (A) Apparatus for delivering stimuli and recording responses to the head-fixed mouse. Not shown is a freely-rotating treadmill, upon which mice walk freely. (B) Typical unconditioned and conditioned responses for a wild-type mouse. Before learning, the mouse generates an eyeblink response to a corneal airpuff unconditioned stimulus (US). After learning over daily sessions spanning 3-12 days, a conditioned stimulus (CS) produces an anticipatory eyelid deflection (conditioned response, CR) that peaks at the time of the US. (C) Cerebellar circuitry involved in delay eyeblink conditioning. (D) Eyeblink conditioning in mouse autism models. From left to right, in order of increasing impairment: *Mecp2*(R308/Y), *Cntnap2+/-*, *Shank3(+/ΔC)*, *Cntnap2-/-*, patDup(15q11-13). Black indicates wild-type controls with SEM in gray. Error bars indicate SEM.

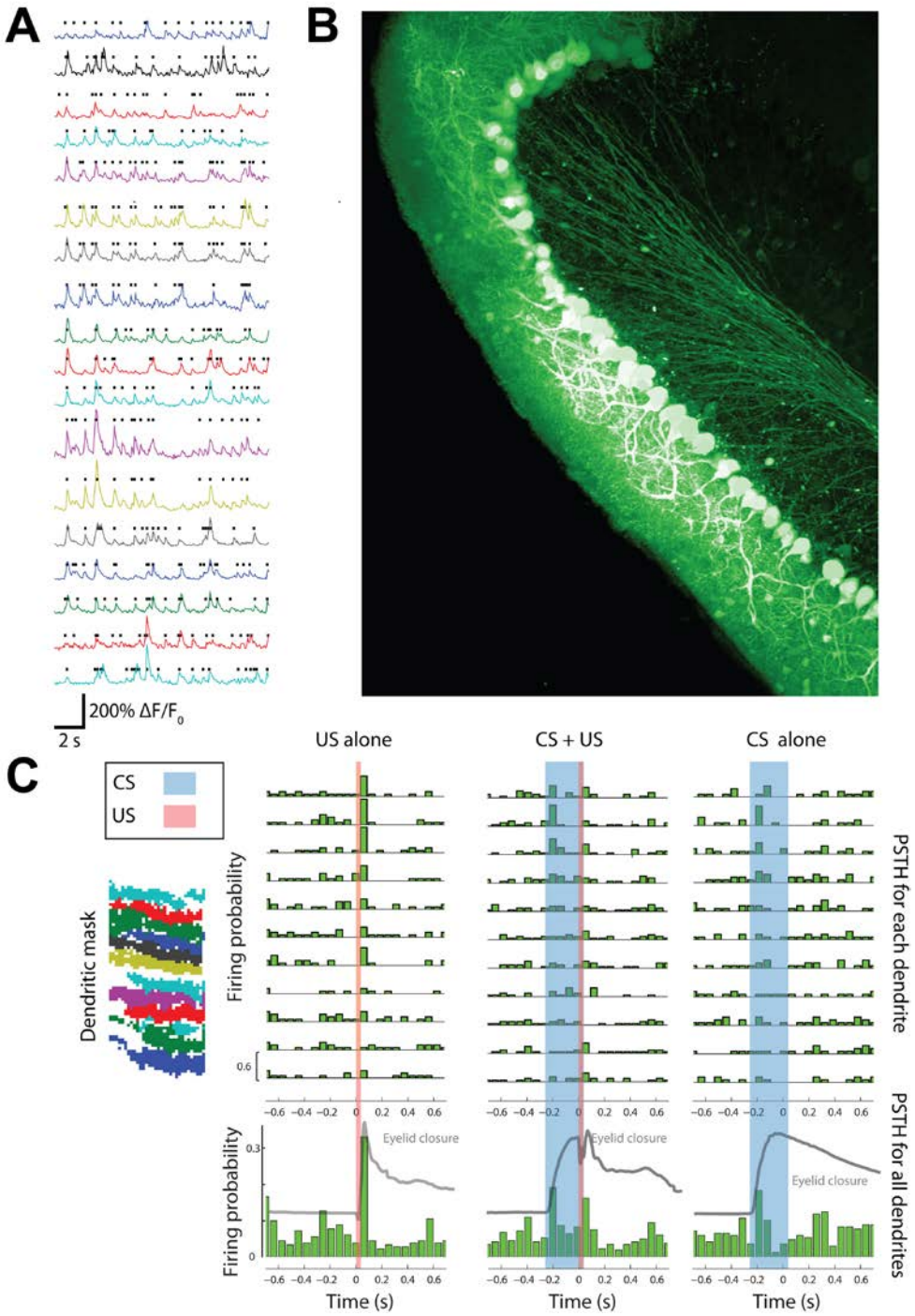

Figure 2. Purkinje cell dendritic imaging. (A) Spontaneous calcium transients in Purkinje cells expressing the calcium indicator protein GCaMP6f in the vermal zone of lobule VI. Black dots indicate calcium transients classified as complex spike-like events. (B) Maximum-intensity projection using two-photon microscopy of Purkinje cells expressing GCaMP6f from the same region imaged in (A). (C) Calcium imaging in Purkinje cells using the synthetic indicator OGB-1/AM in a mouse trained on eyeblink conditioning. *Left:* Regions of interest corresponding to Purkinje cell dendrites. *Right, top:* Peri-stimulus time histograms corresponding to each dendrite for US-alone trials, CS-US paired trials, and CS-alone trials. The vertical red line indicates the US onset and the blue shaded area the CS presentation. *Right, bottom:* Peri-stimulus time histograms for all dendrites pooled. Overlaid as a gray trace is the average time course of eyelid closure for that type of trial.

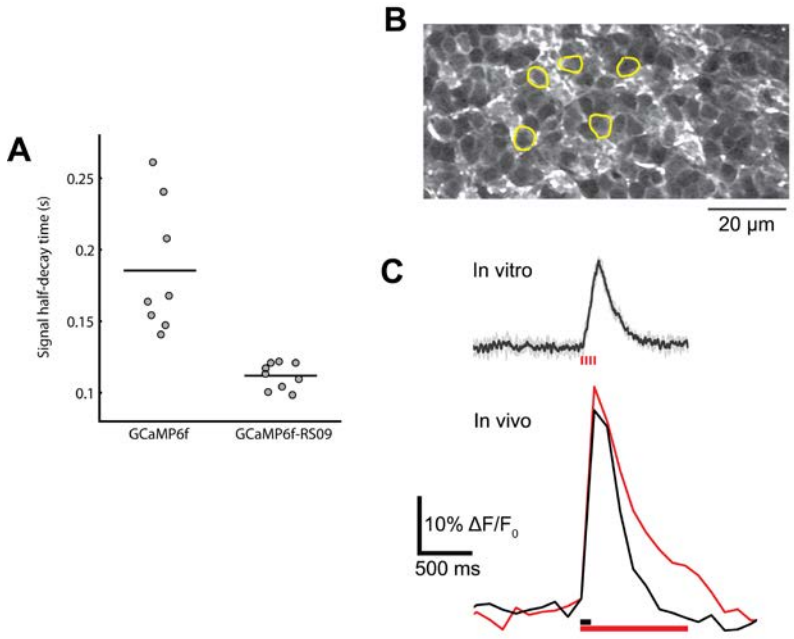

Figure 3. Fast-GCaMP variants and in vivo granule cell imaging. (A) Decay time $t_{1/2}$ for GCaMP6f (from Janelia Farm) and Fast-GCaMP6f-RS09 (from the Wang laboratory) after a brief burst of action potentials in layer 2/3 neocortical neurons recorded in brain slices at 34°C. (B) GCaMP6f expression imaged in vivo in the granule cell layer. Putative granule cells are 5-7 µm wide and have a characteristic excluded nucleus (see also Ozden et al., 2012). (C) *Top,* somatic signal in a single GC in a brain slice in response to a 160-ms burst of 15 mossy fiber stimuli. *Bottom, in vivo* somatic signal from a single GC in cerebellar lobule VI in response to a facial airpuff of 100 ms or 1000 ms duration.

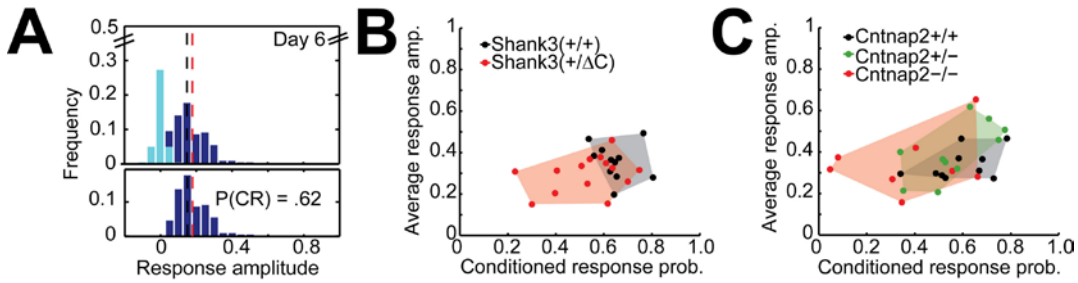

Figure 4. Variability of eyeblink conditioning in autism model mice. (A) Quantal-style analysis to separate CR trials from non-CR trials. Negative-response and zero-response distributions are used to define "failure" events (light blue bars) which are subtracted from the overall response distributions (dark blue) to produce a distribution of CRs. Success rate is defined as the fraction of non-failures, and average CR amplitude is defined from the CR distribution. (B) Average response amplitude and probability in *Shank3* models. *Shank3(+/ΔC)* mice show more inter-individual variability in CR probability than *Shank3(+/+)*, while generating similar-size responses on successful trials. (C) Average response amplitude and probability in *Cntnap2* models. *Cntnap2-/-* mice show more variation in CR probability than *Cntnap2+/-* or *Cntnap2+/+*.

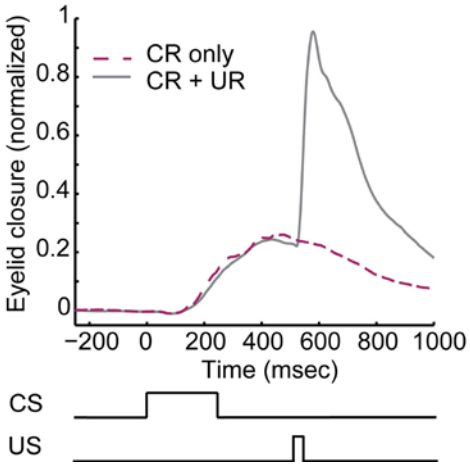

Figure 5. Trace conditioning in head-fixed mice. Example traces from a wild-type animal after trace conditioning. Trace interval, 250 msec. Conditioned stimulus (CS) duration, 250 msec. Unconditioned stimulus (US) duration, 30 msec. Gray trace, response to a CS-US pairing. Magenta trace, CS response.

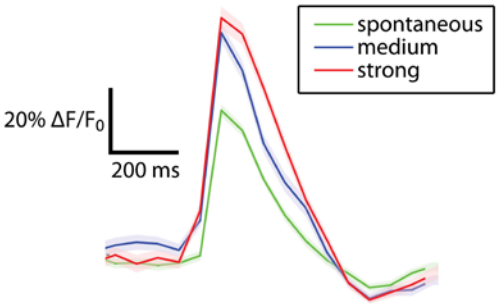

Figure 6. Variation in Purkinje cell dendritic calcium signal as a function of stimulus intensity. Responses to whisker-airpuffs of varying strength. Shaded regions indicate SEM. Data shown is an average across multiple trials in a single PC dendrite expressing GCaMP6f in an untrained mouse.

Principal Investigator Name:	Principal Investigator Institution:	Principal Investigator Email:
Samuel S.-H. Wang	Princeton University	sswang@princeton.edu
Project Title		
Optical approaches to cerebellar circuit function in autism mouse models		

References

Alexander GM et al. (2009) *Neuron* **16**:27-39. Apps R (2000) *Progress in Brain Research* **124**:201-211. Apps R, Garwicz M (2005) *Nature Reviews Neuroscience* **6**:297-311. Argüello PA, Enquist LW, and Wang SS-H (2012) *Society for Neuroscience Meeting Planner* 104.30. Barlow JS (2002) *The cerebellum and adaptive control*, Cambridge Univ. Press. Bangash MA, *et al.* (2011) *Cell* **145**:758-72. Ben-Shachar, *et al.* (2009) *Human Molecular Genetics* **18**:2431-42. Blakemore SJ, Wolpert DM, Frith CD (1998) *Nature Neuroscience* **1**:635-640. Boeckers TM, *et al.* (2001) *Journal of Biological Chemistry* **276**:40104-40112. Boele HJ, Koekkoek SK, De Zeeuw CI (2010) *Frontiers in Cellular Neuroscience* **3**:19. Boudouin SJ, *et al.* (2012) *Science* **338**:128-32. Buckner RL et al. (2011) *Journal of Neurophysiology* **106**:2322-2345. Chadderton P, Margrie TW, Häusser M (2004) *Nature* **428**:856-860. Chettih SN, McDougle SD, Ruffolo LI, Medina JF (2011) *Frontiers in Integrative Neuroscience* **5**:72. Courchesne E, Redcay E, Morgan JT, Kennedy DP (2005) *Developmental Psychopathology* **17**:577-97. Domnisoru C, Kinkhabwala AA, Tank DW (2013) *Nature* **495**:199-204. Eilers J, Augustine GJ, Konnerth A (1995) *Nature* **373**:155-158. Fournier KA, *et al.* (2010) *Journal of Autism and Developmental Disorders* **40**:1227-40. Freeman JH Jr. and Rabinak CA (2004) *Integrative Physiological and Behavioral Science* **39**:180-191. Geisler S and Zahm DS (2005) *Journal of Comparative Neurology* **490**:270-94. Giovannucci A *et al.* (2011) *Society for Neuroscience Meeting Planner* 183.19. Giza J, *et al.* (2010) *Journal of Neuroscience* **30**:14805-16. Kalmbach BE *et al.* (2010) *Journal of Neurophysiology* **103**:2039-49. Klin A, Lin DJ, Gorrindo D, Ramsay G, Jones W (2009) *Nature* **459**:257-61. Kloth AD, *et al.* (2012) *Society for Neuroscience Meeting Planner* 443.21. Kuhn B, *et al.* (2012) *Frontiers in Neural Circuits* **6**:49. Lee and Kim (2004) *Journal of Neuroscience* **24**:3242-50. Limperopoulos C, *et al.* (2012) *Cerebral Cortex* doi:10.1093/cercor/bhs354. McCormick and Thompson (1984) *Science* **223**:296-9. Linden DJ (1999) *Neuron* **22**:661-666. Mathy A. *et al.* (2009) *Neuron* **62**:388-399. Nakatani J. et al. (2009) *Neuron* **137**:1235-1246. Ozden I *et al.* (2012) *PLoS ONE* **7**:e42650. Ohkura M. et al. (2012) *PLoS ONE* **7**:e39933. Palmen SJMC, van Engeland H, Hof PR, Schmitz C (2004) *Brain* **127**:2572-83. Paul A, *et al.* (2012) *Frontiers in Neural Circuits* **6**:37. Peñagarikano O, *et al.* (2011) *Cell* **147**:235-46. Phillipson OT (1979) *Journal of Comparative Neurology* **187**:117-43. Sears LL, Finn PR, Steinmetz JE (1994) *Journal of Autism and Developmental Disabilities* **24**:737-51. Schmahmann JD (2004) *Journal of Neuropsychiatry and Clinical Neuroscience* **16:**367-78. Schneider ER, Civillico EF, Wang SS-H (2013) *Journal of Neurophysiology* doi:10.1152/jn.00925.2012. Shahbazian MD et al. (2012) *Neuron* **35**:243-254. Siegel JJ et al. (2012) *Journal of Neurophysiology* **107**:50-64. Steinlin M (2008) *Cerebellum* **7**:607-10. Steinmetz JE, Lavond DG, Thompson RF (1989) *Synapse* **3**:225-233. Strick PL, Dum RP, Fiez JA (2009) *Annual Review of Neuroscience* **32**:413-34. Timmann D, Drepper J, *et al.* (2010) *Cortex* **46**:845-57. Tobia MJ and Woodruff-Pak DS (2009) *Behavioral Neuroscience* **123**:665-76. Tsai PT, *et al.* (2012) *Nature* **488**:647-51. Vogelstein JT et al. (2010) *Journal of Neurophysiology* **104**:3691-3704. Wang SS-H, Denk W, and Häusser M (2001) *Nature Neuroscience* **3**:1266-1273. Watabe-Uchida, *et al.* (2012) *Neuron* **74**:858-73. Wegiel J. et al. (2013) *Brain Research* **1512**:106-122. Weiss C and Disterhoft JF (2011) *Behavioral Neuroscience* **125**:318-26. Wilber AA and Wellman CL (2009) *Neuroscience Letters* **460**:214-218. Wilber AA et al. (2011) *Neuroscience* **177**:56-65.

Children's Hospital **Harvard Medical School**

Mustafa Sahin, MD, PhD
Associate Professor of Neurology

Children's Hospital, CLSB 13074
300 Longwood Avenue
Boston, MA 02115
Phone: 617-919-4518
Email: mustafa.sahin@childrens.harvard.edu

Samuel Wang, Ph.D.
Department of Molecular Biology and Neuroscience Institute
Princeton University
Lewis Thomas Laboratory, Washington Road
Princeton, NJ 08544

June 20, 2013

Dear Sam,

I write to confirm our collaboration in the area of behavioral testing of autism model mice. This is in support of your Simons Foundation proposal on circuit dynamics to explore cerebellar coding and learning.

My laboratory investigates the normal cellular functions of signaling pathways implicated in neurological disease, with an emphasis on axon growth and guidance. Our research centers upon the proteins affected in tuberous sclerosis complex (TSC) and spinal muscular atrophy (SMA) -- two neurological disorders whose genetic basis is well understood but whose cell biology remains unknown. As you know, recently (Tsai et al. 2012 *Nature* 488:647-651), we reported that early-life cerebellum-specific disruption of Tsc1 leads to autistic-like behavioral phenotypes including abnormal social interaction, repetitive behavior and vocalizations.

We would be glad to work with you to implement these behavioral assays in your laboratory, where you have been working with Shank3 and Cntnap2 models. Our experience, especially that of my postdoctoral fellow Dr. Peter Tsai, should speed your efforts. He and Dr. Aleksandra Badura in your laboratory are already in contact.

I look forward to further conversation and visits.

Yours sincerely,

M. Sahin

Mustafa Sahin

Penn
Arts & Sciences

Department of Psychology

Dr. Samuel S.-H. Wang
Princeton Neuroscience Institute
Princeton University
Princeton, NJ

June 18, 2013

Dear Sam,

As you know, my lab has recently developed a system for eyelid conditioning in head-fixed mice, combining a cylindrical treadmill on which the mice can walk and a high speed camera to measure eyelid movements with millisecond precision. We have been using this system for the last 2 years with excellent results: (1) reliability – 100% of the mice can be successfully conditioned, (2) performance – the vast majority of the mice will achieve >70% conditioned response rate, (3) timing – changing the interstimulus interval results in an adaptive modulation of the timing of the conditioned response. I think that our system will be very useful for the conditioning-related projects in your Simons Foundation proposal.

We have helped set-up one of these eyelid conditioning systems in your lab and have also trained one of your students, Alex Kloth, to use it properly. In addition to its excellent performance, an additional advantage of our eyelid conditioning system is that it is very straightforward to use. Alex has already conditioned a number of mice successfully and I am confident that he will be able to complete the experiments you have proposed to do in your Simons Foundation proposal. Should any problems arise in the future with regards to getting full performance out of the eyelid conditioning system, I would be happy to assist you and help you troubleshoot.

Sincerely,

Javier Medina
Assistant Professor
Department of Psychology
University of Pennsylvania