Conditional deletion of cadherin-13 perturbs golgi cells in the cerebellum and disrupts social and cognitive behaviors

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ABBREVIATIONS

Cdh13	Cadherin-13
ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
ASD	Autism spectrum disorder
CD	Compound discrimination reversal
CDR	Compound discrimination
CHO	Chinese ovary hamster cells
CNS	Central nervous system
CNV	Copy-number variation
DCN	Deep cerebellar nuclei
DR	Dorsal raphe
ECs	Extracellular cadherin domains
EDS	Extra-dimensional discrimination phase
EGL	External granular layer
fcMRI	Functional connectivity MRI
FISH	Fluorescent <i>in situ</i> hybridization
GAD67	Glutamate decarboxylase 67
GPI	Glycosylphosphatidylinositol
IDS	Intra-dimensional shift
IDSR	Intra-dimensional shift reversal
IGL	Internal granular layer
Ins	Insulin cortex
mEPSC	Miniature excitatory postsynaptic current
mIPSCs	Miniature inhibitory postsynaptic currents
ML	Molecular layer
OFC	Orbitofrontal cortex piriform cortex
OVT	Overtraining
DR	Dorsal raphe nucleus
PCL	Purkinje cell layer
PI-PLC	Phosphatidyl inositol-specific phospholipase C
Pir	Piriform cortex
Rhi	Rhinal cortex
RL	Rhombic lip
SD	Simple discrimination
sEPSCs	Spontaneous excitatory postsynaptic current
sIPSCs	Spontaneous inhibitory postsynaptic currents
SNP	Single nucleotide polymorphisms
TTX	Tetrodotoxin
UBCs	Unipolar brush cells
VZ	Ventricular zone
WM	White matter

Abstract

The cerebellum plays important roles in motor coordination and motor learning. In addition to motor-related functions, the cognitive functions and social behaviors in humans, non-human primates and rodents have been linked to the cerebellum. Cognitive and social behavioral impairments have been described in patients who have suffered damages in the cerebellum. Functional brain imaging studies also provide evidence for the contribution of the cerebellum to cognitive and social-related function. Even though Purkinje cells, the primary projection neurons in the cerebellar cortex, have been linked to non-motor function of the cerebellum, relatively little studies have focused on the role of cerebellar interneurons in cognitive and social behaviors.

Cadherin-13 is classified as a member of cadherin superfamily but its function is diverse in different systems. GWAS studies have reported the link between Cadherin-13 to social and cognitive behavioral impairment-related neurological disorders including autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD) and schizophrenia. We found that *Cdh13* is expressed specifically in the internal granular layer (IGL) inhibitory neurons in the cerebellar cortex. To explore the role of Cdh13 and study the function of the cerebellum through manipulation of the expression of Cdh13, we generated *Cdh13* conditional knockout mice mediated by *GlyT2::Cre* in which *Cdh13* was ablated in the cerebellum and piriform cortex. Ablation of Cdh13 in neurons in these regions results in a decrease of the expression of GAD67 or a change of GAD67 localization suggesting the involvement of Cdh13 in maintenance or regulation of the normal function or development of inhibitory synapses. In addition, the inhibitory inputs on Golgi cells reduced in Cdh13

condition mutant mice support the contribution of *Cdh13* to the function or development of inhibitory neurons that form synapses onto Golgi cells. At the behavioral level, loss of *Cdh13* does not affect general motor coordination or locomotor behaviors, but results in impairments in cognitive flexibility and social interactions. Mice lacking *Cdh13* mediated by *GlyT2::Cre* show cognitive flexibility deficits and disrupted social contact pattern concomitant with increase of reciprocal social interactions. Overall, our findings indicate that *Cdh13* is important for inhibitory circuit of the cerebellar cortex and that genetic manipulation in GABAergic neurons in non-executive centers of the brain, such as the cerebellum, may contribute to neurological disorders related cognitive and social behavioral impairments.

Chapter 1. Introduction

Cadherin-13 (T-cadherin): more than just a cell adhesion molecule

Cadherin-13 (Cdh13), also known as H-cadherin or T-cadherin, is expressed in multiple types of tissues including heart, skin, blood vessel and the central nervous system (CNS) (Lee, 1996; Ranscht and Bronner-Fraser, 1991; Rubina et al., 2007; Zhou et al., 2002). Previous studies have determined the diverse expression pattern of Cdh13 in the CNS (Forero et al., 2017; Killen et al., 2017; Matsunaga et al., 2013; Paradis et al., 2007; Poliak et al., 2016; Price et al., 2002; Ranscht and Bronner-Fraser, 1991; Takeuchi et al., 2000), and distinct roles it plays in different brain regions such as regulation of inhibitory transmission in hippocampal neurons and cell density of dorsal raphe neurons, (Forero et al., 2017; Rivero et al., 2015). However, details of how Cdh13 regulates neuronal processes in these brain regions, and whether Cdh13 controls additional processes is still poorly understood.

The structure of Cdh13 is unique from other members of the cadherin superfamily

Most cadherins are single transmembrane proteins with repeat calcium binding extracellular cadherin domains (ECs) and cytoplasmic tails (Figure 1) (Hirano et al., 2003; Takeichi, 2007). Classic cadherins such as E-cadherin and N-cadherin have five ECs (i.e. EC1 to EC5) and mediate cell adhesion mainly through EC1-3 (Figure 1) (Bayas et al., 2006; Chen et al., 2016; Ciatto et al., 2010; Hirano et al., 2003). The intracellular domains of classic cadherins bind with several molecules including β -catenin and p120 and have been shown to interact with the cytoskeleton to influence cell morphology and movement (Takeichi, 2007). α -catenin is known link cadherin to cytoskeleton by binding with β -catenin (Oas et al., 2013). Desmosomal cadherins, such as desmogleins and desmocollins also have 5 ECs and a

cytoplasmic domain (Garrod et al., 2002). The ECs of desmosomal cadherins contribute to the regulation of cell adhesion and the cytoplasmic domains can bind with intermediate filaments with the help of γ -catenin and plakoglobin/desmoplakin (Diane Wickline et al., 2013; Garrod et al., 2002). Protocadherins, similar to desmosomal cadherins, also have ECs and the cytoplasmic regions, but they have six to seven ECs and their cytoplasmic regions are structurally divergent (Figure 1) (Angst et al., 2001; Hirano et al., 2003). Other members in cadherin superfamily also have conserved ECs meanwhile they perform different functions based on variety of structures (Figure 1) (Hirano et al., 2003).

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Figure 1. Various structures of representative members in cadherin superfamily (Hirano et al., 2003).

The extracellular domain of Cdh13 is considered to be similar to other classical cadherins in the cadherin superfamily (Lee, 1996; Ranscht and Dours-Zimmermann, 1991). When Cdh13 was first reported to be expressed in the CNS of chicken embryo, it was initially named T-cadherin, or truncated cadherin (Ranscht and Dours-Zimmermann, 1991). It was classified as a cadherin because the newly identified gene shares high sequence similarity in its extracellular domains with classical cadherins (38%-47%) (Ranscht and Dours-Zimmermann, 1991). Different from the EC1 of type I classic cadherins, the EC1 of Cdh13 does not have a HAV motif (Ranscht and Dours-Zimmermann, 1991). Identical significant amino acids were found in the sequence of the extracellular domains, such as presumed calcium-binding sites, of both Cdh13 and classical cadherins (Ranscht and Dours-Zimmermann, 1991). Therefore, the extracellular domains of Cdh13 are also known as ECs (Ranscht and Dours-Zimmermann, 1991). However, unlike most members of the cadherin superfamily, the cytoplasmic domain of Cdh13 is truncated (Ranscht and Dours-Zimmermann, 1991).

Instead of a cytoplasmic domain which can interact with cytoskeleton or intracellular signaling molecules, Cdh13 is anchored on the membrane with a glycosylphosphatidylinositol (GPI) anchor (Lee, 1996; Ranscht and Dours-Zimmermann, 1991; Tanihara et al., 1994). Furthermore, crystal structure analysis showed that homophilic adhesive interaction between Cdh13 is distinct from classical cadherins (Ciatto et al., 2010). Classical cadherins homodimerize with each other by inserting a conserved tryptophan residues-formed chain into a hydrophobic pocket, termed β -strands swapping (Boggon et al., 2002; Haussinger et al., 2004; Shapiro et al., 1995). However, Cdh13 lacks a swapping chain that consists of tryptophan residues (Ciatto et al., 2010). Instead, part of the hydrophobic residues from EC1 and EC2 patch together to form an X structure without any strands

exchanging (Ciatto et al., 2010). The proposed unique interaction mechanism implies that Cdh13 exerts its influence in a different manner than classic cadherins in adhesion or cell signaling.

Role of Cdh13 as a cell adhesion molecule

Members of the cadherin superfamily have been demonstrated to have adhesion properties that play various roles in cell-cell and cell-substrate interactions (Hirano et al., 2003). During development, classic cadherins such as E-cadherin have been reported to contribute to cell sorting (Halbleib and Nelson, 2006). The cells that express the same type of cadherins tend to aggregate together and be sorted out from other heterogeneous neuronal populations (Nose et al., 1988). Moreover, cadherins influence structural polarity by attaching to specific cell on one side of a particular tissue type (Godt and Tepass, 1998).

Supported by the structural similarities between Cdh13 and classical cadherins, Cdh13 was first hypothesized as an adhesion molecule which mediates cell-cell and cell-matrix adhesiveness (Vestal and Ranscht, 1992). In a cell aggregation study, Chinese ovary hamster cells (CHO) transfected with Cdh13 aggregated more robustly with other Cdh13-transfected cells compared to non-transfected CHO cells (Vestal and Ranscht, 1992). The result suggests an increase in cell aggregation induced by homophilic binding between Cdh13 (Vestal and Ranscht, 1992). However, even though Cdh13 transfection resulted in an increase in aggregation in an aqueous environment, this experiment does not provide evidence that Cdh13 expression increases aggregation in a natural setting which includes both liquid and solid surroundings.

In contrast to the cell aggregation finding, a different study showed that Cdh13 inhibits the adhesion between two populations of cells which endogenously or ectopically express Cdh13. When a layer of endogenous Cdh13 expressed human umbilical vein endothelial cells (HUVEC) is seeded on the top of a layer of L929 cells (a mouse fibroblast cell line), the percentage of HUVECs that adhere to the monolayer of Cdh13 transfected L929 cells is reduced compared to empty vector transfected cell line (Philippova et al., 2003). The result shows that Cdh13 does not simply serve as an adhesion molecule which only serves to enhance the binding between cells in this system (Philippova et al., 2003). Cdh13 may possess signaling recognition properties for interaction with molecules in a repulsive manner.

Cdh13 is implicated in various signaling pathways and may play a role as a signaling molecule through direct or indirect interaction with other molecules. Cdh13 has been demonstrated to serve as a receptor of adiponectin (Hug et al., 2004). Overexpression of Cadherin 13 in endothelial cells causes the enhancement of ribosomal S6 kinase 1 (S6K1) phosphorylation thereby promoting S6K1 activation (Joshi et al., 2005). This implies that Cdh13 may be involved in S6K1-related PI3K pathway. Also, Cdh13 is found to suppress the proliferation of neuroblastoma cells, glioma cells and cutaneous squamous carcinoma cells by affecting the G2/M phase in cell cycle (Mukoyama et al., 2005). Cdh13 has been reported to play a role in epidermal growth factor receptor (EGFR) pathway as well (Kyriakakis et al., 2012). In cutaneous squamous cell carcinoma (SCC), the overexpression of Cdh13 reduces the phosphorylation of EGFR, and silencing of results in an increase of EGFR activity (Kyriakakis et al., 2012). Therefore, even though Cdh13 may act as a cell adhesion molecule to enhance cellular interactions in some system, it inhibits adhesion or contributes to

signaling pathways in other systems. In order to study the function of Cdh13 in the CNS, it is important to consider that in addition to the mechanisms uncovered for Cdh13 outside the CNS, there may be a specific role that Cdh13 plays within the CNS.

Cdh13 contributes to neurite and axon growth in the CNS

Neurite outgrowth and axon guidance are important processes for the assembly of neural circuits during developmental stages or neuronal repairing (Giger et al., 2010; Tessier-Lavigne and Goodman, 1996). These processes are regulated temporally and spatially by the interplay between intracellular or membrane-bound proteins as well as signals in the environment (Russell and Bashaw, 2018; Tamariz and Varela-Echavarría, 2015). Many cell-adhesion molecules have been shown to play important roles during these processes, including NCAM, integrin and N-cadherin (Hansen et al., 2008). The extracellular domains of these cell-adhesion molecules interact with extracellular molecules and then signals back to the cytoskeleton through their intracellular domains (Shapiro et al., 2007). Using this strategy, the extension and the direction of developing axons can be regulated. Despite lacking a cytoplasmic domain, Cdh13 could mediate axon guidance through interactions with environmental factors that contain conserved extracellular cadherin domains.

The effect of Cdh13 in neurite outgrowth and axon guidance was first demonstrated in motor axons (Fredette et al., 1996; Fredette and Ranscht, 1994). The expression pattern of Cdh13 during critical period of motor axons growth suggests that Cdh13 may act as a negative factor for axon growth (Fredette et al., 1996). To obtain direct evidence, this hypothesis was tested *in vitro* in a neurite outgrowth assay (Fredette et al., 1996). The results

from these experiments revealed several principles about how Cdh13 contributes to neurite growth.

First, inhibition of neurite outgrowth is regulated by homophilic interaction between Cdh13. Primary cultured sympathetic neurons are found to express Cdh13 (Fredette et al., 1996). When sympathetic neurons are cultured on CHO substrata, inhibition of neurite growth is found only in CHO substrata transfected with Cdh13 (Fredette et al., 1996). Similarly, independent of whether CHO is transfected with Cdh13, the neurite outgrowth of ciliary ganglion neurons, which don't normally express Cdh13 and of motor neurons at a stage which do not express Cdh13, is not affected. (Fredette et al., 1996). Additional evidence was provided by the disruption of neurite outgrowth inhibition when Cdh13 with homophilic binding site mutation (R14S D140S) was transfected into substrate (Ciatto et al., 2010). Since the inhibitory effect of Cdh13 on neurite outgrowth is only apparent when the protein is present in both cultured neuron and cell substrata, the suppression influence of Cdh13 on neurite outgrowth is likely regulated by homophilic interactions (Ciatto et al., 2010).

Second, Cdh13 in the environment can influence neurite outgrowth without attachment to the membrane by its GPI anchor (Fredette et al., 1996). Without the GPI anchor, the presence of a Cdh13 EC1-5 recombinant protein in the environment alone is sufficient to inhibit neurite outgrowth of Cdh13-expressing neurons (Fredette et al., 1996). The opposite result was observed when Cdh13 was cleaved from membrane by digestion of its GPI anchor with phosphatidylinositol-specific phospholipase C (PI-PLC). In principle, if EC1-5 of Cdh13 alone can perform the same function as the whole protein with GPI anchor, PI-PLC should not block the influence of Cdh13 on neurite growth. These conflicting results could be due to differences in methods of manipulation. Interestingly, results from earlier

experiments showed that the Cdh13 was not simply cleaved from the membrane but completely removed from the environment after treatment with PI-PLC (Fredette et al., 1996). The removal of the Cdh13 could be explained by the washing steps after enzyme treatment resulting in cleavage of Cdh13 from the membrane. In summary, the extracellular Cdh13 that mediates neurite outgrowth inhibition does not need to be attached on a membrane (Fredette et al., 1996). The neurite outgrowth of Cdh13 expressed neurons can be regulated by free-floating peptide consists of Cdh13 extracellular domains (Fredette et al., 1996). These results revealed that the GPI anchor is not critical for Cdh13 to serve act as a factor to guide neurite growth.

The role of Cdh13 on axon growth regulation is also supported by *in vivo* studies (Hayano et al., 2014). Disruptions in axonal projections result from knockdown or overexpression of Cdh13 in rat cerebral cortex at specific developmental stages (Hayano et al., 2014). As a result of staining with home-made antibody, mAb 6C9, Cdh13 is mainly expressed in the deep layer neurons (layer V and VI) at early postnatal stage, but not upper layer neurons (layer II/III and IV). Ectopic expression of Cdh13 in neurons that express markers of upper layer neuron at embryonic stage results in these neurons accumulating in the deep layers and perturbation of axonal projection direction (Hayano et al., 2014). Moreover, knockdown of endogenous Cdh13 in deep layer neurons resulted in changes of their axonal projections (Hayano et al., 2014). This *in vivo* result shows that Cdh13 is critical to axon guidance during early developmental stages in the cerebral cortex (Hayano et al., 2014). Together with *in vitro* result, the involvement of Cdh13 in neurite growth and axon guidance has been identified in multiple regions in the CNS (Ciatto et al., 2010; Fredette and Ranscht, 1994; Hayano et al., 2014). These studies provide evidence that Cdh13 is a key determinant of the development and organization of neural circuits. The involvement of

homophilic interaction during this process is an underlying cellular mechanism that Cdh13 uses to exert its activities.

Roles of Cdh13 as a regulator of synaptic function

The contributions of cadherins to synaptic formation have been reported in studies of classical and nonclassical cadherins (Figure 2) (Giagtzoglou et al., 2009). N-cadherin has been shown important to spine morphogenesis and maintain normal synaptic function (Saglietti et al., 2007) (Tanabe et al., 2006). Loss of protocadherin results in decrease of synaptic puncta (Weiner et al., 2005).

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Figure 2. Processes of synaptic formation and the function of adhesion molecules during synapse formation

(A-C) Stages during synaptic formation. (D-F) The modulation of adhesion molecules during synaptic formation (Giagtzoglou et al., 2009).

In addition to axonal growth, Cdh13 has also been shown to be necessary for synaptic development (Paradis et al., 2007; Rivero et al., 2015). Knockdown Cdh13 in cultured hippocampal neurons *in vitro* leads to a decrease of both excitatory and inhibitory synapses (Paradis et al., 2007). The number of glutamatergic synapses labeled by postsynaptic density protein95 (PSD95) and synapsin was decreased when Cdh13 was knocked down with siRNA (Paradis et al., 2007). This reduction can be rescued by Cdh13 construct that is resistant to RNAi (Paradis et al., 2007). The reduced glutamatergic synapses are, more specifically, AMPA receptor-contained synapses detected by staining synaptotagmin I and AMPA receptor subunit, GluR2 (Paradis et al., 2007). To test whether this decrease is functional-relevant, miniature excitatory postsynaptic current (mEPSC) of transfected neurons were recorded (Paradis et al., 2007). A decrease of both frequency and amplitude of mEPSC is detected. A change in action potential independent EPSCs after *Cdh13* deletion may be caused by disruption in the number of AMPA receptors at synaptic region, on the amount of glutamate in vesicles released from presynaptic area or in the number of synapses formed on the recorded neuron. These results indicate that the knockdown of *Cdh13* disrupts the formation or development of glutamatergic synapses (Paradis et al., 2007). In addition to excitatory synapses, GABAergic synapses were also analyzed. Two pairs of pre- and post-synaptic GABAergic synaptic markers: VGAT/GABA_A receptor subunit $\beta 2/3$ and GAD67/ GABA_A receptor subunit $\gamma 2$ were used to assess the number of GABAergic synapses in control and *Cdh13* RNAi neurons (Paradis et al., 2007).

Knock down of Cdh13 also results in a decrease in the number of GABAergic synapses (Paradis et al., 2007). Therefore, Cdh13 plays an important role in synaptic development and controls the specialization of both pre- and post-synaptic elements.

The role of Cdh13 on regulation of synapses is supported by *in vivo* evidence from the analysis of *Cdh13* knockout mice. By using a ubiquitous Cre dependent Cdh13 knockout mouse, the expression of Cdh13 was analyzed in the hippocampus (Rivero et al., 2015; Schwenk et al., 1995). The expression of Cdh13 was reported to be located at inhibitory synapses and colocalized with presynaptic, rather than postsynaptic molecular markers. To test the functional significance of Cdh13 ablation, the electrophysiological properties of Cdh13 ablated neurons are analyzed. Results show the Cdh13 regulates miniature inhibitory postsynaptic currents (mIPSCs), but not mEPSCs recorded on postsynaptic target, CA1 pyramidal neurons, of Cdh13 ablated neurons (Rivero et al., 2015). Recordings of CA1 pyramidal neurons showed that the deletion of Cdh13 in presynaptic neurons results in an increase of the frequency of mIPSC (Rivero et al., 2015). To determine whether the increased mIPSC frequency is caused by increased vesicle release frequency or the rise of the inhibitory synapse number, paired pulse ratio was tested. Supported by decreased vesicle release from presynaptic inhibitory neurons and increased apical dendritic branches of postsynaptic CA1 pyramidal neurons, this study inferred that the change of mIPSC frequency at the postsynaptic CA1 pyramidal neurons of Cdh13 knockout (*Cdh13*^{-/-}) sections is likely due to the increased number of inhibitory synapses (Rivero et al., 2015).

Both *in vitro* and *in vivo* studies provided ample evidence that Cdh13 plays an important role in the formation and/or maintenance of synapses. However, the strategy employed by Cdh13 to control synaptic function seems different than one employed to

control neurite or axon growth. An *In vitro* study showed that neurite outgrowth was inhibited when Cdh13 was expressed in both neurons and the substrata (Fredette et al., 1996). Results from this study suggest that Cdh13 influences neurite outgrowth through homophilic interaction. However, the *in vivo* study showed that synaptic transmission of inhibitory synapses formed onto CA1 pyramidal neurons in Cdh13 knock out mice was disrupted (Rivero et al., 2015). Because Cdh13 is only expressed in inhibitory neurons, but not CA1 pyramidal neurons, the authors inferred that Cdh13 was only deleted in inhibitory neurons in Cdh13 knockout mice. Therefore, this *in vivo* study suggests that loss of Cdh13 in presynaptic but not postsynaptic cells is sufficient to disrupt synaptic function (Rivero et al., 2015). In other words, this result suggests that Cdh13 may regulate synaptic functions *in vivo* through a heterophilic interactions with other proteins. While Cdh13 has been shown to be important in cellular and subcellular function in neurons, how manipulation of Cdh13 expression and neuronal processes is linked to human disorders and animal behavior is still not well understood.

Cdh13 is linked to neurological disorders with cognitive and social behavioral deficits

The emergence of the function of Cdh13 on neurite outgrowth, axon guidance and synaptic development leads to examination of the link between Cdh13 dysfunction and neurological disorders. In human studies, Cdh13 has been linked to multiple types of psychiatric and neurological disorders diagnosed in diverse population (Rivero et al., 2013). For instance, Cdh13 has been linked to attention deficit hyperactivity disorder (ADHD) by the identification of Cdh13 single nucleotide polymorphisms (SNPs) in subjects with ADHD. (Lasky-Su et al., 2008; Zhou et al., 2008). In the human chromosome, the *Cdh13* gene is located on 16q24 (Lee, 1996). A meta-analysis based on seven genome-wide linkage scans in ADHD individuals determined that bin 16.4, from 16q23.1 to q terminal, is related to ADHD

with a high significance (genome-wide significant or $p < 0.00042$) for P_{SR} and a small P_{OR} around 0.04 (Zhou et al., 2008). Based on 7 Caucasian studies, the result of genomic search meta-analysis shows that a region which includes *Cdh13* on chromosome 16 is linked to high risk for ADHD (Zhou et al., 2008). Shortly after, an SNP of *Cdh13* within intronic region relate to ADHD was identified (Lasky-Su et al., 2008). Genome association analysis on additional ADHD patients reported another SNP fall within *Cdh13* (Neale et al., 2010a; Neale et al., 2010b). Furthermore, more specific cognitive behaviors of ADHD probands were measured to assess the association of *Cdh13* with ADHD. Multiple *Cdh13* SNPs are found related to verbal working memory, visual-spatial working memory and motor inhibition in ADHD patients (Arias-Vasquez et al., 2011). Analysis of subjects grouped by different ages revealed that *Cdh13* is linked to youths who have ADHD (Salatino-Oliveira et al., 2015). The loci of *Cdh13* SNPs were found in introns of human genome between the third and fourth coding or the fifth and sixth coding exon (Arias-Vasquez et al., 2011; Lasky-Su et al., 2008; Neale et al., 2010a; Salatino-Oliveira et al., 2015). The function of *Cdh13* protein may be disrupted through incorrect splicing of *Cdh13* mRNA results from these SNPs (Cooper, 2010).

Other than ADHD, *Cdh13* has been reported implicated in schizophrenia (Borglum et al., 2014; Otsuka et al., 2015). The linkage between *Cdh13* and schizophrenia is supported by GWAS with both European and Asian patients, two different human populations with very different culture, habits and living environment (Borglum et al., 2014; Otsuka et al., 2015). The link between *Cdh13* and autism spectrum disorder (ASD) is also emerging. A rare copy-number variation (CNV) analysis in 1124 ASD families showed a strong association recurrent CNVs which include the deletion of 34 kb exonic region of *Cdh13* (Sanders et al., 2011). By using IQ discrepancy, the ratio between performance IQ and verbal IQ, as

phenotype, region in chromosome 16 include Cdh13 was linked to communication and social deficits considered as an important point for ASD diagnosis (Chapman et al., 2011).

Cdh13 has also been implicated in other neuropsychiatric disorders (Cho et al., 2015; Johnson et al., 2006; Sokolowski et al., 2016; Tiihonen et al., 2015; Treutlein et al., 2009; Uhl et al., 2008). Cdh13 was linked to bipolar depression through enrichment pathway analysis of data from European-ancestry individuals with these disorders (Cho et al., 2015). Moreover, results from a genome-wide association (GWA) study showed that SNPs of Cdh13 may be associated with alcohol dependence in German male population (Treutlein et al., 2009). The association of Cdh13 and alcohol dependence was also supported data of an association genome scanning study which investigated European and African American population (Johnson et al., 2006). In addition, methamphetamine dependence was also related to Cdh13 in a GWA study based on normally positive SNPs investigated in Japanese and Taiwanese populations (Uhl et al., 2008). Finally, GWA studies showed that disruption in the Cdh13 gene may contribute to extreme behaviors like violent and suicidal behavior in a Finland population and a Ukraine population respectively (Sokolowski et al., 2016; Tiihonen et al., 2015). Even though Cdh13 is associated with many different types of neurological disorders, many of these disorders share similarities in the behavioral alterations, including impairment of cognitive function and social behavioral deficits. Thus, Cdh13 may regulate aspects of neural circuits that control cognitive and social behaviors.

The role of Cdh13 in mediating animal behaviors

To examine the link between Cdh13 and neurological disorders, mouse genetics and behavioral analysis have been employed to determine whether ablation *Cdh13* in mice results in similar behavioral deficits observed in human patients (Drgonova et al., 2016; Rivero et al.,

2015). Knockout of *Cdh13* with a ubiquitous Cre mouse line (*CMV::Cre*) results in higher locomotor activity, impairments of cognitive flexibility assessed by Barnes maze and loss of fear memory using fear conditioning assays (Rivero et al., 2015). In addition to cognitive and locomotor behaviors, loss of *Cdh13* is associated with drug reward processing (Drgonova et al., 2016). The conditioned place preference can be induced in control mice with 5, 10, 20 mg/kg doses of cocaine but this can only be induced in *Cdh13*^{-/-} animals with 5mg/kg doses of cocaine (Drgonova et al., 2016). In both studies, *Cdh13* was ubiquitously ablated in all tissues of the mice (Drgonova et al., 2016; Rivero et al., 2015). The widespread localization of Cdh13 expression likely contributes to the range of behaviors deficits observed in complete *Cdh13* ablation. To study the function of Cdh13 in restricted regions in the CNS and understand whether conditional deletion of this gene could lead to behavioral deficits related to neurological disorders, an animal model which permits selective deletion of Cdh13 will need to be generated.

To study the contribution of Cdh13 to cognitive and social related behaviors, the spatial and temporal expression pattern of the gene must first be defined. Details of the specific brain regions and neuronal subtypes that express Cdh13 are informative for a better understanding of how Cdh13 is involved in processing and regulation of cognitive and social-related behaviors. The cerebral cortex is a complex region which has long been presumed to control and process cognitive and social related functions (Bissonette et al., 2008; Insel and Fernald, 2004). However, the cerebral cortex does not work alone (Bullmore and Sporns, 2012). Other brain regions, such as the cerebellum, may modify signals from the cerebral cortex and play critical roles on cognitive function (Schmahmann, 2010).

Function of the cerebellum beyond motor control and motor learning

Clinical evidence supporting the link between the cerebellum and cognitive-related processes

Analysis of the connections between the cerebellum and behavioral deficits from lesion studies, revealed a great deal about the function of the cerebellum (Flourens, 1824; Holmes, 1908; Luciani, 1891; Rolando, 1809). Importantly, studies revealed that lesions in the cerebellum do not stop movement but reduced the motor accuracy and coordination (Rolando, 1809). Moreover, there is growing evidence showing the ablation of the cerebellum results in subtle movement disturbances including dysmetria, deficient arm movement, gait ataxia, eye movement abnormalities and dysarthria (Ito, 2002; Paulin, 1993). From these studies, the cerebellum has conventionally been accepted as the brain region that contributes to motor control and motor learning.

In addition to motor regulation, the cerebellum also contributes to non-motor function. Non-motor behavioral impairments have been documented in adolescent and adult patients with surgeries that resulted in cerebellar damage. A longitudinal study performed in 22-67-year old subjects whose cerebella were damaged by resection, stroke, cerebellitis or cerebellar cortical atrophy shows the disruption of cognitive-affective function including executive function, spatial cognition, language and personality, while the symptoms subsided gradually in some patients after onset (Schmahmann and Sherman, 1998). The impairments of cognitive-affective function were observed in patients who have damage in the cerebellum with different aetiologies including tumor resection, cerebellar agenesis and cerebellar degeneration (Chheda et al., 2002; Leroi et al., 2002; Levisohn et al., 2000). In all these studies, patients who have lesion on posterior lobules and vermis of the cerebellum show obvious cognitive-affective related behavioral deficits, which indicates that executive

function and social behaviors may be controlled by the cerebellum (Chheda et al., 2002; Leroi et al., 2002; Levisohn et al., 2000).

Since cerebellar lesions result in cognitive-affective function, it is therefore not surprising that the disruption to the cerebellum has also been implicated in neurodevelopmental disorders such as ASD, which is associated with cognitive and social behavioral impairments (Carper and Courchesne, 2000; Rogers et al., 2013; Sparks et al., 2002; Wang et al., 2014). A link between ASD and damages within the cerebellum was established from MRI studies, which revealed vermal hypoplasia in the cerebella of ASD patients (3-9 years old) (Carper and Courchesne, 2000; Sparks et al., 2002). Interestingly, patients suffering from other disorders commonly characterized by defects in the cerebellum, such as Dandy-Walker malformation, Joubert syndrome and pontocerebellar hypoplasia also display ASD-like symptoms (Boltshauser, 2004).

Anatomical and functional connectivity supports a role of the cerebellum in Cognitive processing

Cognitive and social-related functions of the cerebellum have similarly been proposed from observations in patients (Schmahmann, 2010). However, which specific region of the cerebellum may be involved in non-motor function is still not clear. In cerebellum somatotopy studies, two mirroring body representations were found in the anterior and posterior lobules of the cerebellum responses to motor activity (Figure 3) (Adrian, 1943; Buckner, 2013). Interestingly, there exists a large region between two body representations that is “blank”. If this is not related to motor functions, could it be relevant for cognitive functions? Resting-state functional connectivity MRI (fcMRI) study provided clues by demonstrating the connectivity between the cerebellum with related cerebral cortical

regions which contributes to regulation of non-sensorimotor functions (Figure 4) (Buckner et al., 2011; Krienen and Buckner, 2009). The non-motor functional mapping on the cerebellum implies the involvement of the cerebellum in cognitive-affective network in the CNS.

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Figure 3. Somatotopic representation of tongue (blue), hand (red) and foot (green) on the cerebellum in fcMRI study (sagittal view)

The anterior lobules are situated more rostrally and posterior lobules are situated more caudally. This figure shows that the representation areas are repetitive in the cerebellar cortex and show a mirror view pattern. In anterior lobules, the foot representation is anterior to the hand and tongue but posterior to the hand and tongue in posterior lobules (Buckner, 2013; Buckner et al., 2011).

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Figure 4. The functional association between the cerebellum and the cerebral cortex

Different functions are represented by different colors. The same color shown on the cerebellum and the cerebral cortex describe the functional connection between these two regions. For example: orange represent cognitive control area, bluish-gray indicate somatosensory network, red describe default network and green shows sensory-motor integration (Buckner, 2013; Buckner et al., 2011).

The link between the cerebellum and cognitive function is also supported by anatomical tracing results which revealed extensive connections between the cerebellum and the cerebral cortex in non-human primates and rodents (Stoodley and Schmahmann, 2010; Suzuki et al., 2012). The cerebellum is linked to the cognitive areas of the cerebral cortex disynaptically: the cerebral cortex communicates with the cerebellum primarily through the pontine nuclei and receives processed signals from the cerebellum through the thalamus (Strick et al., 2009). Non-motor related cortical areas including the prefrontal cortex, posterior parietal cortex, temporal cortex and limbic cortices all indirectly innervate the

cerebellum (Galgiani, 2013; Schmahmann, 2010; Suzuki et al., 2012). Furthermore, the cerebellum forms closed-loop circuits with the basal ganglia through the pontine nuclei and thalamus (Bostan and Strick, 2010).

Representation of non-motor functions in specific lobules of the cerebellum

Although the structure of the cerebellum is seemingly homogenous, the signals it processes and integrates are not the same at different areas (Snider, 1952). Damage to the cerebellum from stroke disrupts motor behavior when the anterior lobules, rather than the posterior lobules of the cerebellum are involved (Schmahmann et al., 2009). Relatively little deficits in motor behaviors are observed when the posterior lobules are damaged in stroke patients (Schmahmann et al., 2009). Tracing and functional mapping in animal models provided more evidence to support that the non-motor signaling processing in the cerebellum is lobule specific (Suzuki et al., 2012; Watson et al., 2009).

Most of the sensorimotor-related information is transferred to anterior lobules of the cerebellum (lobule I-V), and parts of lobule VI and VIII (Kelly and Strick, 2003; Oscarsson, 1965). Instead, lobule VII and lateral extended lobules Crus1 and Crus2 are linked to higher order information processing (Kelly and Strick, 2003; Sasaki et al., 1975). Projections from the cerebellar cortex to the deep cerebellar nuclei (DCN) are also highly organized: the medial nucleus is linked to the limbic cerebellar cortex (cerebellar vermis), interposed nucleus to the motor cerebellar cortex (lobules I-V and a part of lobule VI) and lateral nucleus to the cognitive cerebellar cortex (lobule VII, Crus1 and Crus2) (Chambers and Sprague, 1955a, b; Haines and Rubertone, 1977; Jansen and Brodal, 2011; Schmahmann, 2010; Stoodley and Schmahmann, 2010). This connectivity pattern suggests that specific

information is relayed to specific nucleus of the deep cerebellum respectively (Buckner, 2013; Schmahmann, 2010).

The link between the cerebellar disruption and cognitive-related deficits at the cellular level

Perturbation in the cerebellum resulting in cognitive-related behavioral deficits could be due to alterations in anatomical or physiological properties neurons in the cerebellum. In the cerebellum of ASD subjects, the size and number of Purkinje cells are different from control subjects (Bauman, 1996; Fatemi et al., 2002a; Kemper and Bauman, 2002). Furthermore, the level of glutamate decarboxylase 67 (GAD67), one of a key enzyme for GABA synthesis, is altered in both Purkinje cells and stellate/basket cells of the ASD cerebellum (Fatemi et al., 2002a; Fatemi et al., 2002b; Whitney et al., 2009; Yip et al., 2007, 2008). Therefore, in order to uncover the mechanisms underlying cerebellar-related disorders, and identify potential therapeutic targets, we must first examine consequences of genetically manipulating cerebellar on physiological and morphological properties of circuits and neuronal subtypes in the cerebellum.

The structure and local circuitry of the cerebellum

The cerebellum is hypothesized to control non-motor behaviors by using the same anatomical substrate it uses to regulate motor coordination (Schmahmann, 2010). The similarity of the general structure through all cerebellar lobules lead to the theory: dysmetria of thought (Schmahmann, 2010). Like movement dysmetria, difficulties in reaching a target, thought dysmetria describes a mismatch between thought and behavior (Schmahmann, 2010).

Structure and major components of the cerebellum

The cerebellar cortex is a well-organized structure that consists of three major parts: the vermis in middle and two hemispheres on both sides linked by paravermis region (White and Sillitoe, 2013). In all three regions of the cerebellum, the cerebellar cortex wraps around the DCN and in between these two structures contains white matter which is made up of axonal fiber tracts (Altman and Bayer, 1997). The cerebellar cortex is composed of three laminar layers: molecular layer (ML), Purkinje cell layer (PCL) and internal granular layer (IGL) from outside to inside (Sillitoe and Joyner, 2007). The arrangement of main subtypes of neurons have been studied in the cerebellar cortex: somata of Purkinje cells are aligned in the PCL, which separates the adjacent ML and IGL (Ito, 2006). Cell bodies of the ML interneurons, stellate and basket cells, are distributed along the ML while cell bodies of granule cells, unipolar brush cells and IGL inhibitory neurons reside in the IGL (Barnack and Yakhnitsa, 2008). Apart from the major neuronal subtypes, there are two main types of fibers that enter the cerebellum, namely mossy fibers and climbing fibers that originate from other parts of the central nervous system (Ito, 2006). The neuronal population residing within each laminar of the cerebellum are not isolated from each other. Instead, different subtypes of neurons in the cerebellum receive signals from and target to specific cells to form microcircuits.

Local circuits in the cerebellar cortex provide basis for signal transfer and processing

As the only inhibitory efferent neurons in the cerebellar cortex, Purkinje cells receive afferent signals from mossy fibers and climbing fibers (Altman and Bayer, 1997). In the IGL, granule cells innervated by mossy fibers extend bifurcate axons into parallel fibers to activate Purkinje cells (Figure 5). In the PCL, Purkinje cells receive excitatory signals from climbing fibers originating from inferior olive (Altman and Bayer, 1997). After processing by circuits

in the cerebellar cortex, all signals are sent to DCN via Purkinje cell axons (Husson et al., 2014). The interneurons in the ML and IGL help regulate signals transferred through granule cell-Purkinje cell pathway.

Figure 5. Organization and circuitry within the cerebellum

Inputs to the cerebellar cortex are provided by climbing fibers and mossy fibers. After integration and modification of this information granule cells and cerebellar interneurons, outputs are sent by Purkinje cells from the cerebellar cortex to the DCN. Efferent fibers from DCN neurons send signals calculated by the cerebellum out to other parts of the brain.

Golgi cell, an important inhibitory neuronal subtype that contributes to the cerebellar function

Golgi cells make up an important subtype of IGL inhibitory interneurons (Altman and Bayer, 1997). Golgi cells inhibit granule cells with feedback and feedforward loops (Geurts et al., 2003). In feedback loop, Golgi cells send inhibitory signals to granule cells and in turn, receive excitatory signals from granule cells (Ito, 1984). Granule cells activate Golgi cells with parallel fibers in the ML and ascending fibers in the IGL (Cesana et al., 2013). Approximately 50% of excitatory signals transmitted from granule cells to Golgi cells are contributed by ascending axons in the IGL (Cesana et al., 2013). In feedforward loop, Golgi cells activated by mossy fibers inhibit granule cells in the IGL (Duguid et al., 2015). By performing functions within those loops, Golgi cells regulate cerebellar inputs in the IGL through inhibiting and shaping the firing of granule cells and furthermore, contributes to regulate animal behaviors. Golgi cells responses to sensory stimulus such as tactile stimulus, sensorimotor processes including locomotion, eye movement, forelimb movements and states of slow oscillation generated in neocortex (Heine et al., 2010; Holtzman et al., 2006; Prsa et al., 2009; Ros et al., 2009; van Kan et al., 1993; Vos et al., 2000). Ablation of Golgi cells result in ataxia-like behavior impairments and mild deficits of motor coordination (Watanabe et al., 1998).

Dual GABAergic and glycinergic phenotype: a genetic entry point for Golgi cell manipulation

While most of inhibitory neurons in the cerebral cortex use only GABA as neurotransmitter, Golgi cells in the IGL use both GABA and Glycine for communication (Ottersen et al., 1987; Voogd and Glickstein, 1998). Interestingly, GABA and Glycine contribute to different synaptic activity in Golgi cell-related circuits (Geurts et al., 2003).

Granule cells are inhibited by Golgi cells only through GABA regulated by GABAA receptors but IGL excitatory interneurons, namely, unipolar brush cells (UBCs) are regulated by both neurotransmitters at inhibitory synapses formed with Golgi cells. Glycine is mainly expressed in hindbrain and spinal cord (Bowery and Smart, 2006). Compared to GABAergic synapse, glycinergic synapse-related molecules provide better tools for specifically manipulating cells in hindbrain such as the cerebellum because of their restricted expression (Jursky and Nelson, 1996).

Experimental objectives

Even though *Cdh13* has been studied in the CNS, the role of *Cdh13* in the cerebellum has not been examined. To explore the expression pattern of *Cdh13* in the cerebellum and the contribution of *Cdh13* to the cerebellar circuitry and cerebellar-related behaviors, we generated mice carrying *Cdh13-floxed* alleles and conditionally ablated *Cdh13* with *GlyT2::Cre* mice. By studying consequences of *Cdh13* deletion on electrophysiological and synaptic properties of Golgi cells, we aim to understand the function of *Cdh13* at the molecular level. Furthermore, to explore the possible involvement of the cerebellum in *Cdh13*-related behavioral impairments, we performed a series of behavioral tests to assess both motor and cognitive functions in mice from different experimental groups. To assess the general locomotor ability in these mice, we performed the open field test, marble burying test and gait pattern test. To assess general motor coordination and motor learning, we performed the rotarod test. To examine the cognitive flexibility and social ability, we performed the 2-choice digging task and reciprocal social interaction test, respectively. To ensure that mice have the ability to dig for performing the 2-choice digging task, we separately examined the digging behaviors in mice from different experimental groups. Finally, to determine whether

any results from the reciprocal social interaction test could be due to disruption to general olfactory processing, we tested mice in odor habituation/dishabituation assays.

Chapter 2. Materials and Methods

Mouse strains

Mice used for this thesis: *Cdh13^{fl/fl}* mice, *GlyT2::Cre* (Ishihara et al., 2010) and *Rosa::lox-stop-lox-eYFP* (The Jackson Laboratory) (Srinivas et al., 2001). *GlyT2::Cre* mice was made by the microinjection of a bacterial artificial chromosome (BAC) with *GlyT2* promotor driven Cre recombinase (Ishihara et al., 2010). The recombination ability of Cre activity in *GlyT2::Cre* mice largely recapitulates the endogenous expression of GlyT2 (Ishihara et al., 2010). *Cdh13^{fl/fl}* mice were generated with ES cells from the European Mouse Mutant Cell Repository (Jm8A3.N1), which have *loxP* sites flanking *Cdh13* exon 3. *GlyT2::Cre; Cdh13^{fl/fl}* (*GlyT2-Cdh13^{-/-}*) mice were generated by crossing *Cdh13^{fl/fl}* mice with *GlyT2::Cre; Cdh13^{fl/fl}* mice. For characterizing embryonic stages, breeders were housed together for 8-12h at night, and then male and female mice were separated. Noon of the next day was counted as E0.5 and the following night was considered E1. All experiments were conducted under the approval of the Institutional Animal Care and Use Committee at Nanyang Technological University and A*STAR. Primers used for genotyping: forward 5' TTCACAATGCTGAGCACTTCCCTAG 3'; reverse 5' ATCCTGGGCTACAGGAGGTCTGCTC 3'. The expected size of WT allele is 96bp and that of targeted allele is 308bp.

***In situ* hybridization**

Fluorescent *in situ* hybridization (FISH) and chromogenic *in situ* hybridization were conducted using modifications of previously described protocols (Schaeren-Wiemers and Gerfin-Moser, 1993; Vosshall et al., 1999). The RNA probe was targeted to a sequence fragment on mouse *Cdh13* mRNA. The primer sequences for making the probe were: forward 5' CAACGAGAAGCTGCACTACG 3' and reverse 5' GCGCTAATACGACTCACTATAGGGGGACACCACAATGGACCTCT 3'.

Figure 6. The location of primers for generation of *Cdh13* *in situ* hybridization probe

Ex, exon. Ex3 is the critical exon flanked by loxps in *Cdh13^{fl/fl}* mice.

Before *in situ* hybridization, animals were perfused with PBS and PFA. Perfused brain tissue was post-fixed for 3 hours and frozen in optimal cutting temperature freezing media (OCT) on dry ice. Frozen brain tissue was sectioned to 18 μ m using a cryostat (Thermo Scientific Microm HM550).

All equipment and containers were cleaned with 70% ethanol and RNase Away (Molecular Bioproducts Catalog# 7002). Steps labeled without temperature were performed at room temperature. For conducting chromogenic *in situ* hybridization, tissue was fixed in freshly made PFA (4%PFA in 0.1M PB pH $\approx$ 7.4) at 4°C for 30min and subsequently washed with PBS (1st BASE 10x Phosphate Buffered Saline c/No.BUF-2040-10x4L) three times,

3min each wash. Proteinase K buffer (1µg/mL proteinase K, 5mM EDTA and 50mM Tris pH7.5) was then used to digest tissue for 10min. Tissue was fixed again in 4%PFA for 5min then washed with PBS for another 3min, repeated thrice. To reduce background, acetylation solution (1.2% triethanolamine and 0.26% acetic anhydride) was applied for 10min. After washing tissue in PBS 3 times, 5min per wash, pre-hybridization was performed by incubating tissue in hybridization solution (50% formamide, 5x SSC, 5x Denhardts, 250µg/mL yeast RNA and 500µg/mL herring sperm DNA) at 60°C for at least 2 hours. A humidified chamber with wet filter paper (filter paper that infiltrated by 5xSSC and 50% formamide) was prepared before the next step. After prehybridization, DIG labeled probes were diluted in hybridization solution and heated at 80°C for 5min. Then tissue slices bathed with probes were covered with coverslips or parafilm and incubated overnight (for 16-18 hours) at 60°C in the humidified chamber.

On the second day, coverslips were removed in pre-heated 60°C 5x SSC. Tissues were incubated in 0.2x SSC at 60°C for 30min. Fresh 0.2x SSC was supplemented and incubated at 60°C or another 30min. Later, tissue was bathed in 0.2x SSC for 5min. B1 buffer (0.1M Tris7.5 and 0.15M NaCl) was applied for 5min. Tissues were then blocked with blocking buffer (10% sheep serum in B1 buffer) for 2 hours and submerged in anti-DIG antibody (Roche Anti-Digoxigenin-AP, Fab fragment) prepared by 1:2000 dilution in B1 buffer with 1% sheep serum at 4°C overnight.

Next morning, tissues were washed three times with B1 buffer, 5min per wash. Then equilibrated in B3 buffer (100mM Tris-HCl pH=9.0, 150mM NaCl and 1mM MgCl) for 5min. Finally, 1 drop of Levasimole solution (vector laboratories), 0.66% NBT and 0.33% BCIP was diluted in B3 buffer and the mixture was added to tissues for incubation until

signals were detected on tissue. When the signal was strong enough, reaction was stopped and extra signals were washed with cold Methanol and mounted with Dako Glycergel Mounting Medium (Dako).

FISH protocol was the same as chromogenic *in situ* hybridization before the incubation in B1 buffer on the second day. After B1 buffer, tissue was blocked in blocking buffer, TNB (Perkin Elmer) for 30min. Later, signal amplification was performed with TSA plus fluorescein System (PerkinElmer, NEL741001KT) and TSA plus cyanine 5 System (PerkinElmer, NEL745001KT). 5% H₂O₂ was used for 15min to quench reaction between different labeling processes. Usually, antibodies for weaker probes were used first. After staining, ProLong Gold antifade reagent (life technologies) was used to mount the slices and reduce the chance of signal bleaching.

Immunohistochemistry

Staining of slice-mounted sections

Tissue of which the thickness was 18µm was treated with 0.2% Triton-X in PBS for 8 minutes. Then, the tissue was blocked in blocking buffer [PBS, 0.1% Triton-X (OmniPur), 2% horse serum (Invitrogen)] for at least 2 hours. Tissue was incubated with primary antibody overnight at 4°C. Next day, the tissue was washed in washing buffer [PBS, 0.1% Triton-X (OmniPur)] 3 times, 5min per wash. Later, secondary antibody diluted in blocking buffer was incubated with tissue. At last, tissue was washed again with washing buffer and mounted with mounting reagent [ProLong Gold antifade reagent (life technologies)]. Primary antibodies used in the immunohistochemical staining for slice-mounted sections: mouse anti-GAD67 (1:500, Merck, MAB5406), rat anti-GFP (1:1000, Nacalai Tesque, GF090R), mouse anti-mGluR2 (1:1500,

Advanced Targeting System, AB-N32), rabbit anti-Neurogranin (1:1000, Merck, AB5620).

Analysis with 18 μm mounted sections permits rapid identification of the location of the sections with mouse brain atlas after staining, and preservation of structural integrity, especially when the sections contain several brain regions (e.g. sections of midbrain and hind brain).

Staining of Free-floating sections

40 μm cryosections were used for free-floating staining. Tissue was washed in washing buffer (TBS) and blocked in blocking buffer [TBS, 0.4% Triton-X (OmniPur), 3% donkey serum (Millipore, S30-100ML)]. Primary antibody was incubated for 48 hours at room temperature. After 3 washes, sections were incubated with secondary antibody for 4-6 hours at room temperature. Sections were mounted as above. Primary antibodies used in the immunohistochemical staining for free-floating sections: mouse anti-GAD67 (1:500, Merck, MAB5406). Free-floating staining enable both sides of the tissue sections to be exposed to the antibody.

Staining of neurobiotin

Tissue of which the thickness was 250 μm was fixed with 4% PFA overnight. Then, the tissue was washed in washing buffer [PBS, 0.2% Triton-X (OmniPur)] 5 times, 5 minutes per wash. Tissue was blocked in blocking buffer [PBS, 0.2% Triton-X (OmniPur), 3% horse serum (Invitrogen)] for one hour at room temperature. After that, tissue was incubated with Streptavidin Alexa 633 (1:250, Molecular Probes, S21375) overnight at 4°C. Next day, the tissue was washed in washing 3 times, 10min per wash. Then, tissue was mounted with mounting reagent [ProLong Gold antifade reagent (life technologies)].

Image analysis

All images were collected using a Zeiss confocal microscope (LSM710). For analysis of GAD67 intensity and area, image acquisition parameters were kept the same between control and experimental groups for all tissues. NIH ImageJ (version 1.46r) was used for quantification. Z-stack images were collected on a Zeiss LSM710 confocal microscope (63x objective, numerical aperture 1.4 oil, Plan-Apochromat). By using ImageJ, a 53 μm x 53 μm region was randomly selected as region of interest (ROI). Within the selected ROI, the mean gray values of all stacks were measured. The six images with highest mean gray values were chosen and the mean grey value of the maximum intensity Z-projection of the six images was calculated. For analysis of both the intensity and area of GAD67, we quantified values between 60 and 255 (i.e. grey values below 60 was considered as background and removed before analysis) for conversion of images to binary pictures in which signals were shown by black color. Then, the area of GAD67 was calculated by measuring the area of black regions. The analysis was done for Figure 21-23.

For analysis of YFP⁺ cell diameters, Z-stack images were taken by using a Zeiss LSM710 confocal microscope (20x objective, numerical aperture 0.8, Plan-Apochromat). The longest Feret diameter of the cell was measured after applying maximum intensity Z projection to Z-stack images (Figure 11).

***In vitro* electrophysiology**

Sagittal cerebellar sections (250 μm) of mice aged between 26-35 days were quickly collected at the room temperature. Before use, the sections were incubated at 34°C for 1 hour in oxygenated standard extracellular solution (126 mM NaCl, 24 mM NaHCO₃, 1 mM NaH₂PO₄, 2.5 mM KCl, 2.5 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, 0.4 mM ascorbic acid (pH 7.4/ 95% O₂/5%

CO₂). Whole-cell patch clamp recordings were done at room temperature and followed protocol previously described (Kim et al., 2014). For Golgi cell whole-cell recordings, electrodes (4-7 MΩ) were filled with CsCl-based internal solution (140 mM CsCl, 4 mM NaCl, 0.5 mM CaCl₂, 10 mM HEPES, 5 mM EGTA, 2 mM MgATP, 0.4 mM Na₃GTP (pH 7.3, ~290 mOsm)). Both EPSCs and IPSCs were recorded under voltage clamp with a holding potential of -60 mV and the access resistance was compensated. In the internal solution, neurobiotin (0.5%; Vectorlabs, Burlingame, CA) was added to identify Golgi cell morphology.

Spontaneous IPSCs were measured in the presence of glutamate receptor antagonists. AMPA receptor antagonist, NBQX (10 μM) and NMDA receptor antagonist, D-AP5 (50 μM) were added to block excitatory synaptic inputs (Micu et al., 2006). Similarly, spontaneous EPSCs were measured when GABAergic and glycinergic inputs were blocked by SR95531 (10 μM, GABA_A receptor antagonist) and strychnine (0.3 μM, glycine receptor antagonist). To record miniature IPSCs or EPSCs, tetrodotoxin (TTX; 1 μM) was further added in bath solution. The frequency and amplitude of spontaneous and miniature EPSCs/IPSCs were analyzed in 5 minutes after drug equilibration (5-10 minutes).

Behavioral analyses

During the period of behavioral testing, male mice were group-housed in standard cages and kept under temperature-controlled animal facility (21±2°C) on 12h light/dark cycle with food and water *ad libitum*, if not stated otherwise. Male *GlyT2::Cre; Cdh13^{fl/fl}* mice were used as experimental mice and *Cdh13^{fl/fl}* mice were used as control mice. In our strategy, *GlyT2-Cdh13^{-/-}* mice were generated by breeding *Cdh13^{fl/fl}* females with *GlyT2::Cre; Cdh13^{fl/+}* or *GlyT2::Cre; Cdh13^{fl/fl}* males. All investigators were blind to the genotype of mice during experiments and

behavioral tests were done during the light phase of the day (between 9am and 6pm). Adult male mice were subjected to a battery of tests covering basic behavioral, sensory, motor, cognitive and social functions, by using the order of tests oriented toward increasing invasiveness (Dere et al., 2014). Before examining the performance of the mice in complicated behavioral tasks, whether the mice have the basic ability to achieve the test needs to be assessed (e.g. checking their digging ability in marble test before performing two-choice digging task).

Open field

Spontaneous activity in open field was recorded in a novel grey Perspex arena (60 cmx60 cmx40 cm). Test mouse was put in the center of the arena and allowed to explore in the open field for 7 minutes. The behavior was recorded by a computer-linked video camera. 'Ethovision XT' software (Noldus, The Netherlands) was used to calculate distance and average velocity traveled that can reflect the spontaneous activity. Velocity was measured by dividing total distance by 420 seconds. Stay duration at the corner (four rectangles with the area of 92.8cm² at four corners of the arena) versus center (a circle with the diameter of 17.7cm at the center of the open field) and number of visits were used as read outs of novelty-induced anxiety of the mouse during exploration in the open field arena.

Rota-rod

Rota-rod was done to test motor function, balance and coordination. The equipment comprises a rotating drum (TSE GmbH, Bad Homburg, Germany) and the speed of which was accelerated from 4 rpm to 40 rpm rotations per minute over the period of 5 minutes. Each mouse was placed on the drum individually and the latency of falling from the drum was reported by the automatic detecting system of the machine. To assess motor learning, the Rota-rod test was

performed in 3 trials with 5-15 min intertrial interval on the first day and another 3 trials on the second day with a 24 hour delay. In total, every mouse performed 6 trials.

Odor habituation/ dishabituation test

This test was conducted by sequentially exposing mice to different odors and examining the response of mice to every odor provided (Arbuckle et al., 2015). The sequence used of medium for odors were: water, a non-social odor (rose) and two different social odors (bedding swab from different group-housed cages of the same sex). For non-social odors, the cotton tip was dipped in a solution containing food-grade rose artificial flavor (1:100 dilution, Star Brand, Malaysia). Social odor stimuli were prepared by zigzag swiping the bottom of cages of group-housed mice with a cotton tip. Mice were exposed to each odor in 3 consecutive trials for 2 minutes, with a one-minute inter-trial interval between each trial. The trial was considered as initiation when the cotton tip applicator with odor was presented from the cage lid. Time spent on sniffing the cotton tip was recorded using a stopwatch.

Marble Burying

Digging behavior of mice was tested using the marble burying test. Mice were tested in clean standard rat cages (34.5 cmx56.5 cmx18cm) filled with wood-chip bedding of which the depth is 5 cm. 24 glass marbles were placed on the top of the wood-chip bedding. The marbles were arranged in 6 rows and 4 columns at a distance of 4 cm. Mice were placed in the cage individually in a dim environment and could freely move and act on bedding and marbles for 30 minutes. Number of buried marbles (to 2/3 their depth) was counted.

Resident-intruder test

Inter-male aggression was tested in this study. Group-housed males (C57BL6/JInv; InVivos, Singapore) were used as standard opponents (intruders). Tested mice were single-housed for three weeks before recording. In each trial, one intruder was introduced into the home cage of the single-housed resident. Recording started when the resident was introduced and stopped at first attack. The test is ended if no attack occurred in 600 seconds.

Two-choice digging task

This task was conducted according to previously described work (Bissonette et al., 2008; Chuang, 2014; Garner et al., 2006). This test relies on the digging behavior of mice. Mice were trained to associate the stimuli to reward (Honey Stars, Nestle). While this test uses multimodal tactile stimuli, only one dimension of a stimulus (the texture of the bowl cover) predicts the location of the reward. The other dimension (the texture of the bedding) serves as a factor of distraction.

The training process including habituation, shaping and discrimination learning takes place in a modified perspex box (26.9 cmx18.9 cmx16.3cm; modified from Figure 4) which has three compartments separated by removable plates. The box has a starting compartment at the back and testing compartments in front. Two testing compartments are separated by a fixed plastic plate. There are two independent removable guillotine doors between starting compartment and two compartments (left and right compartments). During the habituation phase, mice were habituated with food reward (Honey star Nestle) and testing apparatus. On first two days, mice were habituated in the maze/box in which all doors were opened and had reward evenly spreading on the floor. Two trials on each day were administered for each mouse and every trial lasted for 10 minutes. Then, shaping phases were performed on the next day. Mice were trained to reach

reward in empty bowls or in bowls with bedding in the maze/box with opened doors. During four shaping phases, same conditions were kept on both side of the maze (e.g. bowl, bedding, position of reward). In the first phase, rewards were put in empty bowls. In the second phase, rewards were put at the top of bowls filled with three quarters of bedding. Animals were able to see the reward before digging. In the third phase, rewards were shallowly buried under the surface of bedding and in the last phase, rewards were deeply buried in the bedding. Animal traveled from starting compartment and finished the trial when food on both side were eaten, or the trial had last for 10 minutes. Four consecutive trials were given for each phase. Corncob bedding were used during shaping phases. The shaping phases were performed in one day. If the mouse had not eaten food on both sides during any of the phases, the failed phase and the subsequent phase(s) will be repeated on the next day. During the whole period of the test, mice were food-deprived, and their body weights were maintained between 85%-95% of free-feeding weight. The discrimination phases started after shaping phases. One digging bowl was placed at the end of each testing compartment. Rewards were buried deeply in the bedding of one of two bowls and animals need to dig to get the reward. Then, mice were trained to associate one specific stimulus (i.e. a type of bowl cover) in a dimension of stimuli (i.e. bowl cover), to reward by the response of digging. Another dimension of stimuli (i.e. types of bedding in bowls) randomly shifted in bowls with different covers between trials independent to reward. Each trial started by the sense of door rising. A trial terminated when tested mice first bury their noses to bedding and initiated digging. The criterion is considered when 8 out of 10 trials were correct in each phase. At least 10 trials were administered even if the animal reached criteria in 8 consecutive trials. A missed trial was recorded when animal did not dig in 5 minutes.

Modified from Garner et al., 2006. Copyright © 2006 by Elsevier

Figure 7. Box designed for two-choice digging task (Garner et al., 2006)

The dimensions of the box we used are shown within the parenthesis.

Table 1. Stimulus-reward contingency

Stage	Correct	Relevant Discrimination (bowl cover)	Irrelevant Discrimination (bedding)
		Reward-related stimuli	Irrelevant stimuli
CD	Paper wrap	Plastic wrap	
		Paper vs. Plastic wrap	Square paper bedding Wood chip bedding
CDR	Plastic wrap	Paper vs. Plastic wrap	Square paper bedding Wood chip bedding
IDS	Aluminum foil	Aluminum foil vs. Velvet wrap	Diamond soft bedding Roll paper bedding
IDS-R	Velvet wrap	Aluminum foil vs. Velvet wrap	Diamond soft bedding Roll paper bedding
OVT	Velvet wrap	Aluminum foil vs. Velvet wrap	Diamond soft bedding Roll paper bedding
IDS II	Bubble wrap	Button-textured vs. Bubble wrap	Mixture bedding Pine cat litter
IDS II-R	Button- textured wrap	Button-textured vs Bubble wrap	Mixture bedding Pine cat litter

First, a simple discrimination (SD) phase was presented: there were two stimuli presented in relevant dimension (i.e. bowl cover: e.g. paper wrap versus aluminum foil), while the bedding was kept the same in both bowls. Afterward, a compound discrimination (CD) phase was given: an irrelevant dimension (i.e. bedding: e.g. woodchip bedding versus paper bedding) were introduced. In this phase, mice were trained to avoid being distracted by irrelevant dimension (bedding) and focus on the relevant dimension (bowl cover). When mice reach the criteria of CD phase, a compound discrimination reversal (CDR) phase was presented. The reward-related stimulus of relevant dimension (previously correct bowl cover) became reward-unrelated and previous reward-unrelated stimulus (previously incorrect bowl cover) led to reward. Reward contingencies were switched from acquisition to reversal. Next, a new group of discrimination

acquisition and reversal was presented: intra-dimensional shift 1(IDS1) and intra-dimensional shift 1 reversal (IDS1R). In this group of phases, a new set of stimuli were introduced for both dimensions (a novel type of bowl cover and bedding) and acquisition and reversal phase were repeated. We introduced an overtraining paradigm with 60 extra trials before proceeding to the second set of intra-dimensional shift (IDS2) and its reversal phase (IDS2R). Discrimination phases were done in one day after shaping phases when the shaping phases was done in one day. If shaping phases were repeated, the discrimination phases were done on the same day with repeated shaping phases. Depending on the performance of mice and difficulty of the phases, discrimination phases could be done in one day or multiple days. Usually mice were tested for 45-60 min on each day. In all phases, the position of the bowl with reward-associated cover shifted randomly between left and right compartment of the maze.

Reciprocal social interaction

This test was done by following protocol described previously (Moy et al., 2004). Mice which have the same genotype were tested pairwise in a neutral arena (an arean with pale grey color) (gray Plexiglas box, 30 cm × 30 cm × 30 cm) for 10 minutes. The social interaction of mice was recorded by camera and the videos were analyzed by a trained experimenter who was “blinded” to mice genotype. The duration and frequency of each type of social interaction were measured including: nose-to-nose (nose: anterior to the ear), nose-to-body and nose-to-anogenital (anogenital: ~7% of the most caudal region of the body) were measured. Nose-to-nose or nose-to-anogenital contact was recorded when the nose of one mouse approach the most rostral or caudal part of the body of the other mouse. The initiation period (first three minutes) of the social interaction was analyzed separately from the rest to study whether social behavior in the novel arena was different from the social behavior when mice have been habituated to the arena. Mice which have the same genotype were tested together to simplify the analysis and to avoid potential

confounding effects such as dominant influence on social interaction behavior of mice have one genotype on the other.

Gait analysis

The methods for gait analysis have been described by previous work (Machado et al., 2015; Mendes et al., 2015). In a special-designed mouse walker, total internal reflection disruption was used to get the details of mice footprints during walking (Mendes et al., 2015). Before the test, mice were habituated to the walker and learned to walk straight all the way to the reward (Honey star Nestle) placed at the end of the walker. Then, gait patterns were recorded when mice walked along the walker. A high frame rate camera at 450 frames/second (fps) was used to record the footprints which were then analyzed with Photoshop CS6. The length of each step was measured. The mice walk through the walker several times during testing period and one trial was collected as one data point.

Statistical Analysis

All experimental data was acquired by experimenters who were unaware of mice genotype. Data were analyzed by GraphPad Prism version 6&7 (GraphPad Software, La Jolla, CA, USA). Mann-Whitney *U*-test, analysis of variance (ANOVA) including posthoc test were used to analyze trials effects, genotype and interaction effects of genotype.

Chapter 3. Results

Expression pattern of *Cdh13* in the cerebellar cortex

Cdh13 is expressed in many regions of the central nervous system such as dorsal raphe (DR), spinal cord, hippocampus and cerebral cortex (Forero et al., 2017; Hayano et al., 2014; Poliak et al., 2016; Rivero et al., 2015). In those regions, the expression of *Cdh13* is in different types of neurons including excitatory neuron, inhibitory neuron, interneuron and projection neuron (Hayano et al., 2014; Rivero et al., 2013). The widespread localization of *Cdh13* suggests diverse functions of the gene and challenge of the deduction of its function in one brain region from studies of other regions. A study on human tissue has reported the expression of *Cdh13* in the cerebellum (Takeuchi et al., 2000). To explore more about *Cdh13* function in the cerebellum, we first examined the expression of *Cdh13* by using *in situ* hybridization during developmental stages (Figure 8, A-H). *Cdh13* signals is not detected at embryonic day 14 (Figure 8, A, B). From embryonic day 18 onwards, *Cdh13* staining is detected in the cerebellum (Figure 8, C-H). To determine the cerebellar neuronal subtype that expresses *Cdh13* at different development stages, a GABAergic neuronal marker, *GAD67* was used. After comparing *Cdh13* signals with *GAD67* signals, we found that at embryonic day 18, both *Cdh13* and *GAD67* were detected in the white matter layer of the cerebellum, where inhibitory neuronal precursors are generated (Schambra, 2008; Sotelo and Rossi, 2013; Zhang and Goldman, 1996) (Figure 8, C, D). From postnatal day 10 onwards, the expression of *Cdh13* was concentrated in the IGL and its pattern was similar to the pattern of *GAD67* at the same layer (Figure 8, E-H). This pattern is consistent with those previously described by others: *GAD67* stains stellate/basket cells in the ML, Purkinje cells in the PCL and granular inhibitory neurons in the IGL (Gourfinkel-An et al., 2003). Hence, the *in situ* hybridization results indicate that *Cdh13* is expressed in internal granular layer inhibitory neurons in the

cerebellum from postnatal day 10 onwards. Double fluorescent *in situ* hybridization, also revealed that ~ 99.86% *GAD67*⁺ cells express *Cdh13*, but no *Cdh13* signal was detected in the PCL and the ML (Figure 8, I-K; Table 2). 412 neurons on 20 sections of 3 animals were analyzed. In conclusion, *Cdh13* is mainly expressed in granular layer GABAergic inhibitory neurons in the cerebellar cortex although we have not ruled out the possibility that *Cdh13* is expressed in excitatory neurons or glia.

Figure 8. The expression of *Cdh13* is found in a subset of GABAergic neurons in the cerebellar IGL. (A-H) Expression pattern of *Cdh13* at different development stages. Scale bar=200 μ m (A-H), 20 μ m (I-K). At E14, *Cdh13* can hardly be found in the WM layer and VZ (A) and limited *GAD67* signals was detected (arrows; B). At E18, the expression of *Cdh13* and *GAD67* was detected in the cerebellar WM layer (arrows; C, D). At postnatal stages P10-100, the signals of both *Cdh13* and *GAD67* were presented in the cerebellar IGL (arrows; E-H).; PCL, Purkinje cell layer; EGL, external granular layer; RL, rhombic lip; IGL, internal granular layer; ML, molecular layer; WM, white matter; VZ, ventricular zone. (I-K) The expression of *Cdh13* and *GAD67* are found colocalized in the cerebellar IGL by using Double fluorescent *in situ* hybridization (arrows; I-K). The signals of *Cdh13* colocalized with *GAD67* in the IGL, but not ML or PCL. (white arrows; I, J, K).

Table 2. Analysis of the density of *Cdh13*⁺ and *GAD67*⁺ cells using FISH at P10

	<i>Cdh13</i> ⁺ neurons%	<i>GAD67</i> ⁺ neurons%
<i>GAD67</i>	99.86±0.14 (N=3; n=20)	100 (N=3; n=20)
<i>Cdh13</i>	100 (N=3; n=20)	82.52±0.36 (N=3; n=20)

N, number of animals; n, number of brain sections. Data presented as mean $\pm$ sem.

Selective deletion of *Cdh13* with *GlyT2::Cre* mice

*Generation of the *Cdh13*^{fl/fl} mice*

To explore the function of *Cdh13* in the cerebellum, we employed the Cre/loxP strategy in mice. ES cells were purchased from the European Mouse Mutant Cell Repository. By collaborating with Dr. Weiping Yu, ES cells (www.eummc.org) were used to generate mice carrying alleles of *Cdh13* with loxp flanked exon 3 (*Cdh13*^{fl/fl}) (Figure 9, A, C). According to the design, Cre-mediated recombination will excise exon3 of *Cdh13* from genomic DNA before translation, which will result in the loss of functional *Cdh13* protein in

the cell because of, perhaps, misreading during translation (Dickinson et al., 2016). PCR primers were designed to distinguish between wildtype (*Cdh13*^{+/+}), heterozygotes (*Cdh13*^{fl/+}) and homozygotes (*Cdh13*^{fl/fl}) mice. We selected representative mice for each genotype and used their tissues for PCR analysis. In the result of PCR analysis, the DNA of wildtype mouse only has WT *Cdh13* alleles, *Cdh13*^{fl/fl} mouse only has targeted alleles and *Cdh13*^{fl/+} mouse has both alleles. (Figure 9, C-D). Based on these results, we proceeded to generate conditional knockout mice.

Figure 9. Generation of *GlyT2::Cre; Cdh13*^{fl/fl} mice

(A) method for *GlyT2::Cre; Cdh13*^{fl/fl} mice generation. The critical exon, *exon 3* on *Cdh13* allele is flanked by *loxP*. *Cdh13* conditional knockout mice were generated by ablating the critical exon with *GlyT2::Cre* recombinase. (B) Breeding strategy: *GlyT2::Cre* was provided by male breeder. (C) Primers designed for PCR analysis. PCR analysis was performed after FRT (flippase recognition target) cassette was removed by the recombination driven by flippase (Flp) (D) Difference of genomic DNA of WT, *Cdh13*^{fl/+} and *Cdh13*^{fl/fl} mice is reported by PCR analysis.

Analysis of the GlyT2::Cre recombination pattern in the cerebellum

Glycine transporter 2 (GlyT2) is known as a transporter of inhibitory neurotransmitter, glycine, which is mainly expressed in the hindbrain and the spinal cord (Jursky and Nelson, 1996). To delete *Cdh13* in the hindbrain regions, we acquired *GlyT2::Cre* mice and crossed them with *Cdh13^{fl/fl}* mice. The *GlyT2::Cre*-mediated recombination has been reported in the cerebellar cortex, but whether *GlyT2::Cre* has the ability to recombine in cerebellar Golgi cells is still unclear (Ishihara et al., 2010). To analyze the recombination pattern of *GlyT2::Cre* in the IGL of the cerebellar cortex, we crossed the Cre line with a reporter line, *Rosa26.lsl.eYFP*. Two markers of Golgi cells, one of two main groups of internal granular layer inhibitory neurons, metabotropic glutamate receptor 2 (mGluR2) and Neurogranin (NG) were stained on the section of *GlyT2::Cre; Rosa26.lsl.eYFP* mice. GFP was used to stain EYFP protein. The rat-anti GFP antibody we used had been used and demonstrated for detection of YFP by others (Sokolowski et al., 2015). We found YFP expression in all three layers of the cerebellar cortex (Figure 10, A). In the IGL, most of mGluR2⁺ and NG⁺ neurons were stained with GFP (Figure 10, B, C, F, G). Our analysis showed that about 98.32 % of mGluR2⁺ neurons are GFP⁺ and about 94.86 % of NG⁺ neurons are GFP⁺ (308 cells were analyzed; Table 3). A previous study showed that the number of total mGluR2⁺ neurons and NG⁺ neurons is 93% of Golgi cells and 62% of total IGL inhibitory neurons (Simat et al., 2007). The rest of GFP⁺ neurons can be Golgi cells or other IGL inhibitory neurons (i.e. Lugaro cells). From here, we knew that more than 85% of Golgi cells have *GlyT2::Cre* recombination ($94.86\% \times 93\% \sim 98.32\% \times 93\% = 88.22\% \sim 91.44\% > 85\%$).

Since GlyT2 expressed in glycinergic inhibitory neurons, we speculated that *GlyT2::Cre* was also expressed in glycinergic inhibitory neurons. However, the expression of

GlyT2::Cre recombinase was not always 100% overlap with the pattern of the GlyT2 protein expression even though the same promoter was used (Ishihara et al., 2010; Jursky and Nelson, 1996). In order to confirm that *GlyT2::Cre* mice results in recombination only in IGL inhibitory neurons, we observed the size of GFP⁺ neurons. IGL inhibitory neurons (i.e. Golgi cells and Lugaro cells) are larger than excitatory neurons (i.e. granule cells and unipolar brush cells). The soma size of Golgi cell, Lugaro cells, granule cell and unipolar brush cells are 10-20µm, 10-13µm, 4-6µm and 8-10µm respectively (Barmack and Yakhnitsa, 2008; Simat et al., 2007). In GFP⁺ group, both small and big neurons were found. In short, the recombination of *GlyT2::Cre* recombinase occurred in most of Golgi cells in the IGL, while IGL inhibitory neurons are not the only group of neurons in which *GlyT2::Cre* recombined. Because *Cdh13* was only expressed in IGL inhibitory neurons, the recombination of Cre had no functional effect on neurons in which no *Cdh13* is expressed in *GlyT2::Cre; Cdh13^{fl/fl}* mice.

Figure 10. *GlyT2::Cre* recombined in Golgi cells in the IGL of the cerebellar cortex.

(A-C) *GlyT2::Cre* recombination is detected in Neurogranin⁺ (yellow arrows; A, C), mGluR2⁺ (red arrows; A, B) and Neurogranin⁺mGluR2⁺ neurons (white arrows; A, B, C) of an adult *GlyT2::Cre*; *Rosa::lox-stop-lox-eYFP* mouse. Purkinje cell soma are highlighted by asterisks. (E-G) a zoomed-in region from figure A-C. YFP expression can be found in most of the mGluR2⁺ cells (F) and Neurogranin⁺ cells (G). Scale bar=100 μ m (A-G)

Table 3. Analysis of the *GlyT2::Cre* recombination

GFP ⁺ neurons%	
mGluR2 ⁺	98.32 $\pm$ 0.83 (N=1; n=5)
NG ⁺	94.86 $\pm$ 1.3 (N=1; n=5)

N, number of animals; n, number of brain sections. Data presented as mean $\pm$ sem.

Figure 11. Histogram of the diameter of YFP⁺ cells in *GlyT2::Cre* recombined reporter mice x-axis, maximum Feret diameter of YFP⁺ cells; y-axis, relative frequency.

*Analysis of the ablation of *Cdh13* in the forebrain, midbrain and the cerebellum*

GlyT2::Cre was found recombined in PC, however, GlyT2 was not found in PC in previous studies (Ottersen et al., 1987). The *GlyT2::Cre* recombination was not restricted to Glycinergic neurons or neurons expressed GlyT2 (Figure 10, A-G) (Ishihara et al., 2010). In this case, the expression pattern of GlyT2 is not recapitulated by the recombination pattern of *GlyT2::Cre* mice. Therefore, we analyzed the *GlyT2::Cre* recombination pattern in not only the cerebellum but also the cerebral cortex and the midbrain. The recombination of *GlyT2::Cre* has been briefly reported in the cerebral cortex (Ishihara et al., 2010). To further analyze *GlyT2::Cre* recombination and the deletion of *Cdh13*, we performed quantitative

analysis of *GlyT2::Cre* recombination and *Cdh13* expression in the forebrain and the midbrain. We first determined brain regions that have both *GlyT2::Cre* recombination and *Cdh13* expression in *GlyT2::Cre; Cdh13^{fl/fl}* mice. The number of *Cdh13*⁺ neurons in the cerebellar cortex was significantly reduced in *GlyT2::Cre; Cdh13^{fl/fl}* mice compared to *Cdh13^{fl/fl}* mice (For quantification, the background was calculated and removed in each image according to the gray value. Only cells with more than 50% coverage were counted.) (Figure 12, A, B; Figure 13, A; Table 4). Most of the *Cdh13* was ablated in the IGL but not all. The remaining *Cdh13* signal in *GlyT2::Cre; Cdh13^{fl/fl}* mice could be due to incomplete recombination mediated by *GlyT2::Cre* or the non-specific binding of our *in situ* probe with exon 2 of *Cdh13* mRNA after deletion of exon 3. The number of *Cdh13*⁺ neurons in the IGL of control mice was 9 times more than that of mutant (Figure 13, A). In other words, the expression of *Cdh13* in around 90% of IGL inhibitory neurons was removed. This result was consistent with data shown in colocalization experiment on *GlyT2::Cre* recombination and Golgi cell marker (Figure 10, B, C, F, G; Table 3).

Figure 12. Ablation of *Cdh13* in the IGL of the cerebellum mediated by *GlyT2::Cre*

(A-B) In the IGL of the cerebellar cortex of an adult control mouse, *Cdh13* expression is detected by using *in situ* hybridization (A), but in an adult *GlyT2::Cre; Cdh13^{fl/fl}* mouse, *Cdh13* signal is not found (B).

Corresponding area (Ai-Aiii and Bi-Biii) from control and *GlyT2-Cdh13^{-/-}* mouse (red boxes in A, B).

Scale bar=1000 μ m (A, B), 100 μ m (Ai-Biii, Ai-Biii). *GlyT2-Cdh13^{-/-}*: *GlyT2::Cre; Cdh13^{fl/fl}*.

We found a few structures in the forebrain and midbrain which had both *GlyT2::Cre* recombination and *Cdh13* expression, including orbitofrontal cortex (OFC), piriform cortex (Pir), insulin cortex (Ins), rhinal cortex (Rhi), hypothalamus and dorsal raphe nucleus (DR) (Figure 13-18). Except for the Pir, the number of *Cdh13⁺* cells in these regions was not reduced in *GlyT2::Cre; Cdh13^{fl/fl}* mice (Figure 14, 16, 18). The number of *Cdh13⁺* cells in the Pir of control mice is 1.75 times more than in mutant mice (Figure 14, B). In short,

besides a slight reduction of *Cdh13* expression in the Pir, *Cdh13* expression is not ablated in most of the forebrain and midbrain regions of *GlyT2::Cre; Cdh13^{fl/fl}* mice (Figure 15-20).

Figure 13. In the cerebellum and the piriform cortex, *Cdh13* was ablated in *GlyT2-Cdh13^{-/-}* mice. (A-B) A reduction in the number of *Cdh13⁺* cells in the cerebellum (A) and piriform cortex (Pir, B) is found. Scale bar=200 μ m. *GlyT2-Cdh13^{-/-}*: *GlyT2::Cre; Cdh13^{fl/fl}*.

Figure 14. The statistical analysis of *Cdh13*⁺ cells in the cerebellum and the piriform cortex of *Cdh13*^{fl/fl} mice and *GlyT2-Cdh13*^{-/-} mice

(A-B) in the cerebellum (A) and piriform cortex (Pir, B) the number of *Cdh13*⁺ cells reduced.

Numbers were normalized by the whole area of structures. *GlyT2-Cdh13*^{-/-}: *GlyT2::Cre*; *Cdh13*^{fl/fl}.

Data presented as mean $\pm$ sem. See also Table 4.

Figure 15. In the orbital frontal cortex, the insular cortex and the rhinal cortex, the *Cdh13* was not removed in *GlyT2-Cdh13^{-/-}* mice.

(A-C) Analysis of the number of *Cdh13*⁺ cells in forebrain regions with both *GlyT2::Cre* recombination and *Cdh13* expression. There is no change in the number of *Cdh13*⁺ cells in the orbital frontal cortex (OFC, A), insular cortex (Ins, B) and rhinal cortex (Rhi, C) of *GlyT2::Cre; Cdh13^{fl/fl}* mice compared to *Cdh13^{fl/fl}* mice. Scale bar=200 μ m. *GlyT2-Cdh13^{-/-}*: *GlyT2::Cre; Cdh13^{fl/fl}*.

Figure 16. Analysis of the number of *Cdh13*⁺ cells in several regions in forebrain

(A-C) No significant difference in the number of *Cdh13*⁺ cells was found in the orbital frontal cortex (OFC, A), insular cortex (Ins, B), rhinal cortex (Rhi, C) of *Cdh13*^{fl/fl} and *GlyT2::Cre; Cdh13*^{fl/fl} mice.

Numbers were normalized by the whole area of structures. *GlyT2-Cdh13*^{-/-}: *GlyT2::Cre; Cdh13*^{fl/fl}.

Data presented as mean $\pm$ sem. See also Table 4.

Figure 17. *Cdh13* was not deleted in the midbrain regions where have both *Cdh13* expression and *GlyT2::Cre* mediated recombination.

(A-B) In the hypothalamus (A), and the dorsal raphe nucleus (DR, B), no reduction of *Cdh13* expression was found in *GlyT2::Cre; Cdh13^{fl/fl}* mice compared to *Cdh13^{fl/fl}* mice. Scale bar=200 μ m. *GlyT2-Cdh13^{-/-}*: *GlyT2::Cre; Cdh13^{fl/fl}*.

Figure 18. Cell-counting result in the hypothalamus and dorsal raphe nucleus of *GlyT2::Cre; Cdh13^{fl/fl}* mice and *Cdh13^{fl/fl}* mice.

(A-B) The number of *Cdh13*⁺ cells is similar in the hypothalamus (A), and the dorsal raphe nucleus (DR, B) of *GlyT2::Cre; Cdh13^{fl/fl}* mice and *Cdh13^{fl/fl}* mice. Numbers were normalized by the whole area of structures. *GlyT2-Cdh13^{-/-}*: *GlyT2::Cre; Cdh13^{fl/fl}*. Data presented as mean $\pm$ sem. See also Table 4.

Figure 19. More regions in forebrain and midbrain have either *Cdh13* expression or *GlyT2::Cre*-mediated recombination.

(A-D) The expression of *Cdh13* is not ablated in these regions: thalamus (A); hippocampus (B); PF, parafascicular thalamic nucleus (C); SNR, substantia nigra, reticular part (D). Scale bar=200μm.

Figure 20. Other regions have either *Cdh13* expression or *GlyT2::Cre*-mediated recombination but no *Cdh13* deletion.

(A-L) No ablation of *Cdh13* is found in these regions: mPFC, medial prefrontal cortex (A-OC; Acb, accumbens nucleus (D-F); VTA, ventral tegmental area (G-I); SC, superior colliculus (J-L). Scale bar=200μm.

The expression or localization of inhibitory synaptic protein is reduced in *GlyT2::Cre; Cdh13^{fl/fl}* mice

Cell adhesion molecules have been shown to regulate synaptic formation and physiology *in vitro* and *in vivo* (Paradis et al., 2007; Rivero et al., 2013). They act as receptors, adaptors or binding factors to assemble synaptic junctions (Washbourne et al., 2004). Members from cadherin superfamily such as N-cadherin and protocadherins have been reported to be important for maintaining synaptic functions (Aiga et al., 2011; Bruses, 2006; Washbourne et al., 2004). We first set out to address whether Cdh13, a member of the cadherin superfamily, regulates synaptic processes in the cerebellum.

Previous studies have shown that knock-down of *Cdh13* disrupts the development of both excitatory and inhibitory synapses in hippocampal neurons (Paradis et al., 2007; Rivero et al., 2015). To study the function of Cdh13 in the cerebellum, we stained the cerebellar cortex with GAD67. GAD67 known as one of the main isoforms of glutamate decarboxylase which help synthesize GABA at presynaptic regions and the cytosol (Esclapez et al., 1994). The expression of GAD67 is regulated by neuronal activity (Lau and Murthy, 2012). By staining GAD67 on control and *GlyT2::Cre; Cdh13^{fl/fl}* tissue sections, we found that the intensity and area of GAD67 expression was reduced by ~26%-30% and ~47%-49% respectively in the Crus2 and lobule 9 IGL of *GlyT2::Cre; Cdh13^{fl/fl}* section (Figure 21-22, A-D). The inhibitory presynaptic regions in the IGL consist of Purkinje cell collateral terminals, Golgi cell axonal terminals, Lugaro cell axonal terminals and nucleocortical terminals from dcn (Ankri et al., 2015; Eyre and Nusser, 2016; Guo et al.). The reduced expression and/or localization of GAD67 may reflect the changes of synaptic strength or numbers of one type

or multiple types of terminals originate among these resources. The result may indicate that expression of *Cdh13* in IGL inhibitory neurons is necessary for maintaining a normal range of inhibitory activity in the cerebellum of *GlyT2::Cre; Cdh13^{fl/fl}* mice.

Figure 21. The expression/localization of GAD67 is reduced in the IGL of *GlyT2::Cre; Cdh13^{fl/fl}* mice. (A-D) The expression of GAD67 in the IGL of lobule IX (LIX) and Crus2 in the cerebellum of *Cdh13^{fl/fl}* (A, B) and *GlyT2-Cdh13^{-/-}* mice analyzed by immunohistochemistry method (C, D). Scale bar=20 μ m. *GlyT2-Cdh13^{-/-}: GlyT2::Cre; Cdh13^{fl/fl}*.

Figure 22. Quantification of the GAD67 signal in Crus 2 and lobule IX (LIX)

(A,C) The Intensity of GAD67 signal in Crus2 and LIX (Crus2: control= 19.09 ± 1.14 , *GlyT2-Cdh13*^{-/-}= 13.7 ± 0.99 , U=99, p=0.002, Mann-Whitney U-test, N=3, n=20-22; LIX: control= 17.55 ± 1.08 , *GlyT2-Cdh13*^{-/-}= 13.2 ± 0.72 , U=103, p=0.0003, Mann-Whitney U-test, N=3, n=22-24). (B, D) Quantification of the area coverage of GAD67 in Crus 2 and LIX (Crus2: control= 202.9 ± 21.87 , *GlyT2-Cdh13*^{-/-}= 107.5 ± 19.04 , U=103, p=0.003, Mann-Whitney U-test (two-tailed), N=3/group, n=20-22/group; LIX: control= 147.3 ± 21.42 , *GlyT2-Cdh13*^{-/-}= 70.3 ± 12.5 , U=100, p=0.0002, Mann-Whitney U-test, N=3/group, n=22-24/group). Control: *Cdh13*^{fl/fl}, *GlyT2-Cdh13*^{-/-}: *GlyT2::Cre*; *Cdh13*^{fl/fl}. N, number of animals; n, number of fields. Data presented as mean $\pm$ sem. See also Table 2.

Interestingly, the change in GAD67 expression/localization was not detected in all lobules. In lobule 2 and lobule 4/5, the amount of GAD67 in mutant mice is comparable with that of control (Figure 22-23). The decrease in GAD67 is mostly concentrated on posterior lobules

which have been reported to be important for cognitive-related function and social behaviors (Schmahmann, 2010).

Figure 23. Quantification of the intensity of GAD67 in 5 different lobules of the cerebellum
L2: lobule 2; L4/5, lobule 4/5; L9, lobule 9; Crus1, lobule Crus1; Crus2, lobule Crus2. *GlyT2-Cdh13*^{-/-}:
GlyT2::Cre; Cdh13^{fl/fl}. *P<0.01, Mann Whitney U test. N=3, n=20-22; N, number of animals; n, number of
fields. Data presented as mean ± sem.

Loss of *Cdh13* results in disruption of inhibitory inputs of cerebellar Golgi cells

The expression of *Cdh13* is detected in IGL inhibitory neurons in the IGL and the expression of GABAergic synaptic marker in the IGL of select lobules is reduced in *GlyT2::Cre; Cdh13*^{fl/fl} mice, but how this reduction contributes to function of IGL inhibitory neurons remains unclear. To explore the functional impact of ablation of *Cdh13* on IGL inhibitory neurons, Dr. Kim Jinsook (Augustine Lab) recorded the electrophysiological

properties of a major class of IGL inhibitory neurons, Golgi cell, by using whole-cell patch clamp technique. Golgi cells were distinguished from other cell types in the IGL (i.e. granule cells, unipolar brush cells and Lugaro cells) according to cell size, range of IPSC frequency and characteristics of TTX-dependent IPSCs (Barmack and Yakhnitsa, 2008; Dugué et al., 2005; Hirono et al., 2012). In the IGL, major neuronal subpopulations include inhibitory neurons consisting of Golgi cells and Lugaro cells and excitatory neurons consisting of granule and unipolar brush cells (Geurts et al., 2003). The size of Golgi cells (10-20µm) is larger than granule cells (4-6µm) and unipolar brush cells (8-10µm) (Barmack and Yakhnitsa, 2008). To distinguish the Golgi cell from Lugaro cells, the IPSC frequencies of Golgi cells and Lugaro cells were used as a parameter (Golgi cell < 10Hz; Lugaro cell >10Hz) (Dugué et al., 2005; Hirono et al., 2012). Additionally, Golgi cells have TTX-independent spontaneous IPSCs but Lugaro cell primarily have TTX-dependent IPSCs (Dugué et al., 2005; Hirono et al., 2012). First, the spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the environment of AMPA and NMDA receptor antagonist. The amplitude of sIPSCs was not changed, but a decrease in sIPSCs frequency was measured (Figure 24, C, E). To further assess whether the reduction is action potential dependent or not, we incubated cerebellar slices in tetrodotoxin (TTX), and recorded mIPSCs on Golgi cells. The results were consistent with sIPSCs. Frequency of mIPSCs but not amplitude decreased on *GlyT2::Cre*; *Cdh13^{fl/fl}* Golgi cells compared to control (Figure 24, D, F). Additionally, no difference on input resistance was detected between Golgi cells of *Cdh13^{fl/fl}* and *GlyT2::Cre*; *Cdh13^{fl/fl}* mice. In short, the frequency of action potential-independent IPSCs is reduced in *Cdh13* ablated Golgi cells, but the amplitude is not disrupted.

Similar experiments were performed to measure spontaneous EPSCs (sEPSCs) and mEPSCs. Instead of using glutamatergic receptor antagonists, GABAergic and Glycinergic

receptor antagonists were used. However, neither frequency nor amplitude of both sEPSCs and mEPSCs were changed in Golgi cells of *GlyT2::Cre; Cdh13^{fl/fl}* mice (Figure 24, G-J). Hence, the inhibitory inputs to Golgi cells were disrupted by deleting *Cdh13* but excitatory inputs did not change.

Overall, the decrease of inhibitory inputs may influence the basal activity of Golgi cells, which further affect the signal integration on Golgi cell soma. Since Golgi cells are critical to granule cell regulation through feedforward and feedback inhibitory loops, disruption of Golgi cell spontaneous activity may influence on signal transferring or processing in the cerebellar cortex (Ito, 2006).

Figure 24. Loss of *Cdh13* impairs postsynaptic properties of Golgi cells.

(A) Whole-cell patch clamp recording of Golgi cells in the IGL labeled with Neurobiotin. (B) Examples of traces of spontaneous postsynaptic current and miniature postsynaptic current of Golgi cells in *Cdh13^{fl/fl}* and *GlyT2-Cdh13^{-/-}* mice (P26-35). (C-F) Analysis of inhibitory postsynaptic current of Golgi cells in the IGL of control and *GlyT2-Cdh13^{-/-}* mice shows reduced sIPSC frequency in *GlyT2-Cdh13^{-/-}* mice (C, $p=0.005$, Mann-Whitney U-test) and decreased mIPSC frequency of Golgi cells in *GlyT2-Cdh13^{-/-}* mice compared to control (D, $p=0.002$, Mann-Whitney U-test). No differences in amplitude of both sIPSC and mIPSC were found between control and *GlyT2-Cdh13^{-/-}* Golgi cells (sIPSC amplitude, $p=0.5$ Mann-Whitney U-test; mIPSC amplitude $p=1.0$ Mann-Whitney U-test) (E, F). (G-J) Excitatory postsynaptic current in control and *GlyT2-Cdh13^{-/-}* Golgi cells. sEPSC frequency and amplitude did not change in *GlyT2-Cdh13^{-/-}* Golgi cells compared to control (G, I, sEPSC frequency $p=0.9$, Mann-Whitney U-test; sEPSC amplitude $p=0.6$ Mann-Whitney U-test). In *GlyT2-Cdh13^{-/-}* Golgi cells, both amplitude and frequency of mEPSC were not different from that of control Golgi cells (H, J, mEPSC frequency $p=1.0$ Mann-Whitney U-test; mEPSC amplitude $p=0.9$ Mann-Whitney U-test). Data presented as mean $\pm$ SEM. P26-35 mice. N=7-9/group, n=10-14/group. Scale bar=20 μ m. N, number of animals; n, number of cells. See also Table 3.

Locomotor function and anxiety-level are not affected by deleting *Cdh13* with *GlyT2::Cre*

Testing baseline locomotor activity is critical for rodent behavioral studies. The lack of assessment of the baseline activity in mice could lead to misinterpretation of results obtained from behavioral tests such as anxiety (Wahlsten, 2011). Furthermore, baseline activity can change when mice have been trained for multiple lab-based behavioral training (Wahlsten, 2011). Therefore, before any behavioral tests commenced, we first tested baseline activity of our *GlyT2::Cre; Cdh13^{fl/fl}* mice. Open field has been used for testing baseline locomotor activity because movement of mice in the novel arena is mainly driven by

exploration (Broadhurst, 1957, 1958). To test the general locomotor function and baseline activity, a square arena was used for open field test and video was recorded by camera set above the arena. The movement of mice was tracked automatically by tracing software ('Ethovision XT' software, Noldus, The Netherlands). Our analysis revealed no significant difference in baseline activity between control and *GlyT2::Cre; Cdh13^{fl/fl}* mice (Figure 25, A-C). Both traveled distance and velocity were comparable between control and *GlyT2::Cre; Cdh13^{fl/fl}* mice in the open field arena (Figure 25, A, B). Our data showed that loss of *Cdh13* does not affect gross locomotor function, exploration behavior and baseline locomotor activity.

Figure 25. Deletion of *Cdh13* in the cerebellum has no impact on general locomotor activity.

(A, B) *GlyT2-Cdh13^{-/-}* mice show comparable distance traveled (A) and velocity (B) during 7 minutes of free locomotion in an open arena. N=12-19/group (C) Digging behavior was not influenced by *Cdh13* deletion. Data presented as mean±SEM. Adult male mice, N=10-19/group. N, number of mice. See also Table 7.

In addition to locomotor activity, open field test can also assess the anxiety level of the mouse (Wahlsten, 2011). In an open field with uniform color, incursion into the center is anxiety related. With high anxiety level, mice tend to avoid moving into the center (Lipkind et al., 2004). To assess the anxiety level of *GlyT2::Cre; Cdh13^{fl/fl}* mice, we measured the duration of stay in the center and the corner of the open field and also the number of times that mice visited the center of the open field. Heatmaps and trajectories were generated according to the duration of stay in a particular region of the open field (Figure 26, C). The heatmap and trajectory for each group are representative examples derived from one mouse for each genotype. The warmer colors indicate that mice spent a longer duration in the area while cooler colors indicate a shorter duration. Similar to control mice, *GlyT2::Cre; Cdh13^{fl/fl}* mice stayed longer at the corner of a square arena compared to the center. There was also no difference in the times of center visit between control and *GlyT2::Cre; Cdh13^{fl/fl}* (Figure 26, A, B). Together, *GlyT2::Cre; Cdh13^{fl/fl}* mice showed normal level of center avoidance and deletion of *Cdh13* did not seem to change the anxiety level of the animals.

Figure 26. Deletion of *Cdh13* in the cerebellum has no impact on anxiety level.

(A) Open field anxiety-related readouts revealed no genotype differences in preference for corner/peripheral zone or center zone. (B) Analysis of the number of visits to the center zone of the open field. (C) No genotype differences were detected in exploration of the open field as illustrated by the heat map of locomotion tracks. N=12-19/group. N, number of animals. Data presented as mean $\pm$ sem. See also Table 7.

Cdh13 is expressed in cerebellar IGL inhibitory neurons and *Cdh13* can be deleted in IGL inhibitory neurons through *GlyT2::Cre* -mediated recombination (Figure 12, B). The genetic manipulation of IGL inhibitory neurons permits the analysis of the behavioral consequences in mice. The role of Golgi cell in locomotor behavior has been assessed by chemo-genetic ablation of Golgi cells (Watanabe et al., 1998). In this study, immunotoxin induced cell ablation occurred in Golgi cells in which IL-2R α was conditionally expressed driven by mGluR2 promotor (Watanabe et al., 1998). Locomotor impairments were observed in mice with Golgi-cell ablation (Watanabe et al., 1998). On the fourth to fifth day after Golgi cell ablation, animal failed to coordinate well on the rotarod at a speed of 10rpm. Even though this impairment on the rotarod at 10rpm recovered gradually and eventually disappeared on the tenth day, Golgi cell-ablated animals still performed poorly on the rotarod at 20rpm (Watanabe et al., 1998). Similar phenotype was observed in beam balance test. The motor coordination defect on the balance beam due to Golgi cell-ablation subsided around 10-11 days after ablation in the simpler task, but no recovery was found in more challenging tasks. Together, these results indicate that Golgi cells are important for motor coordination and the impairment of motor coordination caused by Golgi cell ablation is irreversible.

When ablation of *Cdh13* was found in IGL inhibitory neurons, we asked whether the removal of a gene in IGL inhibitory neurons including Golgi cells would result in the same

behavioral deficits. To test this, rotarod test was done with control and *GlyT2::Cre; Cdh13^{fl/fl}* mice (Figure 27, A). Mice were tested for two days and three trials on each day. The rotarod ran with the acceleration from 4 to 40 rpm/min. In our result, *GlyT2::Cre; Cdh13^{fl/fl}* mice can perform as well as control mice and both control and *GlyT2::Cre; Cdh13^{fl/fl}* mice showed a tendency of improvement from the first trial to the sixth trial which implied a learning process (Figure 27, A). Unlike the ataxic footprint pattern reported in mice with Golgi cell ablation (Watanabe et al., 1998), *GlyT2::Cre; Cdh13^{fl/fl}* mice showed normal footprint pattern and stride length (Figure 27, B, C). Even though the distance between steps of *GlyT2::Cre; Cdh13^{fl/fl}* mice were a little bit shorter than control mice, the p value was not small enough to reject the null hypothesis. Furthermore, the conditional knockout of *Cdh13* also did not affect gross motor behavior such as digging (Figure 25, C). Thus, compared to Golgi cell ablation, genetic manipulation of Golgi cells did not change the general motor behavior and walking pattern of the mice.

Figure 27. Deletion of *Cdh13* in the cerebellum has no impact on general motor coordination and gait.

(A) In multiple trials on a rotating rod, *GlyT2-Cdh13*^{-/-} mice display similar performance to that of control mice. (B) *GlyT2-Cdh13*^{-/-} mice show a tendency of making shorter strides during walking (p=0.06, Mann-Whitney U-test). (C) Comparison of foot prints between control and *GlyT2-Cdh13*^{-/-} mice. N=10-16/group. N, number of mice. Data presented as mean ± sem. See also Table 7.

Deficits in cognitive flexibility of *GlyT2::Cre; Cdh13*^{fl/fl} mice after overtraining

Human studies showed that posterior lobules of cerebellum contribute to executive functions (Rosenbloom et al., 2012). Recent animal studies have linked the cerebellum to cognitive flexibility by ablating Purkinje cells and granule cells in the cerebellar cortex and neurons in inferior olivary nucleus (Dickson et al., 2017). Cognitive flexibility is an important part of executive functions (Purves et al., 2013). Executive functions represent cognitive processes required for selecting/controlling behaviors to achieve goals (Purves et al., 2013). Functions such as attention, cognitive flexibility, plan-making, working memory and decision making are considered executive functions (Logue and Gould, 2014; Robbins, 1996). To achieve a goal, animal first need to focus on the task with attention, then form proper rules according to the goal. Simultaneously, monitoring system and working memory help the engagement of the rules to reach the goal (Purves et al., 2013). Cognitive flexibility is one of the processes for establishing and modifying rules (Purves et al., 2013). Although ablation of cells in the cerebellum has been linked to impairments of cognitive flexibility, the molecular mechanism of cerebellar-mediated cognitive flexibility deficiency is still unclear. *Cdh13* has been implicated in disorders with the phenotype of executive function deficits including ASD, ADHD and schizophrenia, which suggests that *Cdh13* might serve as a link between the cerebellum and executive functions by mediating cognitive flexibility (Barkley, 1997; Craig et al., 2016; Orellana and Slachevsky, 2013). Furthermore, we showed that removing *Cdh13* led to changes of chemical

and electrophysiological characteristics of the synapses in the cerebellar cortex (Figure 21-24). Therefore, *Cdh13* can be a candidate molecule which is involved in the cerebellum-related executive function.

We tested cognitive flexibility of *GlyT2::Cre; Cdh13^{fl/fl}* mice by using a two-choice digging task, which includes different discrimination phases with switched contingencies (acquisition or reversal) (Bissonette et al., 2008; Chuang, 2014; Garner et al., 2006). Cognitive flexibility in mice can be tested when there is a reversal switch in existing objects (i.e. a switch from an acquisition phase to a reversal phase) or a switch from existing objects to new objects (i.e. a switch from one set of bowl cover and bedding to another set of bowl cover and bedding). Mice were trained to associate texture of bowl cover with reinforcer (Honey star Nestle). During the test, trained mice need to make a selection between two bowls with different covers. Reward can only be received when a correct selection was made. Digging behavior was considered as response behavior. The trial was over when mice started digging in one bowl and simultaneously, the guillotine door to another bowl was closed. During the test, eight phases were given in sequence: simple discrimination (SD), compound discrimination (CD), compound discrimination reversal (CDR), intra-dimensional shift 1 (IDS1), intra-dimensional shift 1 reversal (IDS1R), overtraining (OVT), intra-dimensional shift 2 (IDS2) and intra-dimensional shift 2 reversal (IDS2R). The irrelevant dimension (i.e. different bedding) was introduced after SD (Figure 28, A). Our result showed that ablation of *Cdh13* affect mice behavior in two-choice digging test. This impairment was strengthened after OVT phase, especially in the first phase after OVT. The *GlyT2::Cre; Cdh13^{fl/fl}* mice made more errors to reach the criteria in IDS2 phase compared to control mice (Figure 28, D). By comparing the phases before and after overtraining, we found that overtraining did not affect the behavior of control mice but affected the behavior of *Cdh13* knockout mice (Figure

28, D). The average number of errors made by *Cdh13* conditional mutant mice was increased after overtraining, while average number of errors made by control mice did not change, which indicated that the deletion of *Cdh13* resulted in cognitive flexibility deficits (Figure 28, D, E). Together, we found that the conditional ablation of *Cdh13* with *GlyT2::Cre* disrupts cognitive flexibility-related executive behavior after overtraining.

A Two – choice digging task: Experimental design

B Trials to criterion across all stages

C Effects of overtraining on trials to criterion

D Effects of overtraining on performance errors

E Effects of overtraining on IDS1/IDS1R and IDS2/IDS2R

Figure 28. Overtraining in two-choice digging task reveals deficit in cognitive flexibility of *GlyT2-Cdh13*^{-/-} mice.

(A) Experimental design and set of stimuli presented in the two-choice digging task: the texture of the outer bowl as the relevant dimension that gave cue for the reward, while the type of the digging media was the irrelevant dimension. (B) Both experimental groups were able to complete all 7 stages of the task, however *GlyT2-Cdh13*^{-/-} mice need more trials to complete the task (repeated measure 2-way ANOVA, interaction $F(6, 90)=0.6298$; $p=0.706$; genotype $F(1, 15)=4.766$; $p=0.0453$; task stage $F(6, 90)=3.005$; $p=0.0101$; CD vs. CDR, $p=0.8893$; IDS1 vs. IDS1R, $p=0.9989$; IDS2 vs. IDS2R, $p=0.8115$; Tukey's multiple comparisons test).

(C) Cumulative performance across all stages before and after overtraining showing higher number of cumulative trials of *GlyT2-Cdh13*^{-/-} mice compared to control mice (repeated measure 2-way ANOVA, Interaction $F(1, 15)=0.5237$; $p=0.4804$; Genotype $F(1, 15)=23.90$; $p=0.045$; OVT $F(1,15)=4.766$; $p=0.0002$). (D) Effects of overtraining on cognitive performance, measured in terms of errors made to reach criterion: average of performance errors of control (left graph: control mice, $p=0.69$, Wilcoxon matched-pairs signed rank test) and *GlyT2-Cdh13*^{-/-} mice (middle graph: *GlyT2-Cdh13*^{-/-}, $p=0.0059$, Wilcoxon matched-pairs signed rank test) prior to and after overtraining and *GlyT2-Cdh13*^{-/-} mice made more errors at IDS 2 stage, commenced directly after overtraining (right graph: $p=0.02$, Mann-Whitney U-test). (E) For *GlyT2-Cdh13*^{-/-} mice, average of performance trials and errors of IDS1 and IDS1R stages was significantly different from that of IDS2 and IDS2R stages (trials, $p=0.014$, Wilcoxon matched-pairs signed rank test; errors, $p=0.014$, Wilcoxon matched-pairs signed rank test). No difference was found in control mice (trials, $p=0.84$, Wilcoxon matched-pairs signed rank test; errors, $p=0.69$, Wilcoxon matched-pairs signed rank test). Data presented as mean $\pm$ SEM. Adult male mice, N= 6-11/ group. N, number of animals. See also Table 4.

Conditional deletion of *Cdh13* disrupts reciprocal social interaction.

Social interaction is an important behavior in daily life of social animals including humans and rodents (Beery and Kaufer, 2015). Social behavior deficits have been considered as a phenotype in multiple neurological disorders such as ASD and schizophrenia (Woodbury-Smith et al., 2010). However, social signal processing in brain is a complicated procedure, which is still not clearly understood. *Cdh13* has been linked to social behaviors alteration in ASD and violence in human genetic studies (Sanders et al., 2011; Tiihonen et al., 2015) (S. Rozen and H.S. Je, unpublished). This raises the possibility that disruptions in the *Cdh13* gene or *Cdh13* signaling contributes to deficits in social signal processing.

For rodents, pheromone, one of the most critical factors that affect social behavior is detected by related sensory organ and processed by relevant brain regions (Luo et al., 2003). The failure to detect or distinguish scent cues may result in impairment of social behavior. To test whether the odor recognition functions of olfactory system in *GlyT2::Cre; Cdh13^{fl/fl}* mice are intact or not, we first performed odor test (Arbuckle et al., 2015). The mice were exposed to non-social and social cues and each odor was tested three times. Both control and mutant mice showed dishabituation between different odors and gradual habituation when kept being exposed to the same odor (Figure 29). Multiple comparisons of the results from *Cdh13^{fl/fl}* mice showed significant difference between non-social odor and social odor (One-way repeated measures ANOVA, $F(2.258, 15.81) = 8.182$, $p=0.0029$; R3 vs. S1-1, $p=0.0182$, Tukey's multiple comparisons test). The difference between non-social odor and social odor was also observed for *GlyT2::Cre; Cdh13^{fl/fl}* mice (One-way repeated measures ANOVA $F(3.8, 49.4) = 13.89$, $p<0.0001$, R3 vs. S1-1, $p=0.0005$, Tukey's multiple comparisons test). These results indicates the dishabituation occurred non-social and social odor for both *Cdh13^{fl/fl}* and *GlyT2::Cre; Cdh13^{fl/fl}* group. Gradual habituation was more prominent when

Cdh13^{fl/fl} mice were exposed to the first social odor (One-way repeated measures ANOVA, $F(2.258, 15.81) = 8.182$, $p=0.0029$; S1-1 vs. S1-3, $p=0.0094$, Tukey's multiple comparisons test). The habituation exhibited a trend in *GlyT2::Cre; Cdh13^{fl/fl}* group but did not reach statistical significance (One-way repeated measures ANOVA $F(3.8, 49.4) = 13.89$, $p<0.0001$, S1-1 vs. S1-3, $p=0.0585$, Tukey's multiple comparisons test). When mice were exposed to other odors, a decreasing trend was detected among three trials of the same odor, but no significant difference was found for both *Cdh13^{fl/fl}* and *GlyT2::Cre; Cdh13^{fl/fl}* mice. Moreover, *GlyT2::Cre; Cdh13^{fl/fl}* mice showed more preference to social odors compared to non-social odor when the odors were presented for the first time which was similarly observed in control mice (One-way repeated measures ANOVA for R1, S1-1 and S2-1: *Cdh13^{fl/fl}* mice, session $F(1.339, 9.37)=11.07$, $p=0.0058$, individual $F(7, 14)=1.498$, $p=0.2458$; R1 vs. S1-1, $p=0.0027$; R1 vs. S2-1, $p=0.4424$; S1-1 vs. S2-1, $p=0.0724$; Tukey's multiple comparisons test; *GlyT2::Cre; Cdh13^{fl/fl}* mice, session $F(1.843, 23.96)=24.12$, $p<0.0001$, individual $F(13, 26)=3.34$, $p=0.0043$; R1 vs. S1-1, $p<0.0001$; R1 vs. S2-1, $p=0.2927$; S1-1 vs. S2-1, $p=0.0008$; Tukey's multiple comparisons test) (Figure 19). Thus, similar to control, *GlyT2::Cre; Cdh13^{fl/fl}* mice are able to distinguish social odors from non-social odors.

Figure 29. Loss of *Cdh13* in Golgi cells does not influence olfaction.

Responses to nonsocial and social odors of *GlyT2-Cdh13^{-/-}* mice are comparable with that of control mice (repeated measure 2-way ANOVA *genotype* $p=0.2$ *session* $p<0.0001$; *genotype x session* $p=0.99$; *interaction* $F(11,220)=0.26$, $p=0.99$; *genotype* $F(1,20)=0.475$; $p=0.5$; *sessions* $F(11,220)=19.74$; $p<0.0001$). W, water; R, rose; S1, the first social odor; S2, the second social odor. N=8-14/group. N, number of animals. Data presented as mean $\pm$ sem. See also Table 9.

After we found that the social odor recognition was intact in *GlyT2::Cre; Cdh13^{fl/fl}* mice, we did reciprocal social interaction test to study social interaction behavior of *GlyT2::Cre; Cdh13^{fl/fl}* mice (Figure 30). Two mice which have the same genotype were put together in a novel square arena. The two mice tested in one given experiment have never encountered each other before the test. Duration, frequency and latency of interaction between two mice were recorded. Interestingly, the duration and frequency of interaction between *GlyT2::Cre; Cdh13^{fl/fl}* mice was more than that between control mice (Figure 30, B,

C). Also, the latency to first contact of *GlyT2::Cre; Cdh13^{fl/fl}* mice was less than control (Figure 30, B). We analyzed the duration and frequency of interaction in first three minutes and found that this phenotype initialized when mice were put into a novel environment and can maintained without being affected by context habituation (social interaction in the rest 7 minutes: count (#): *Cdh13^{fl/fl}* mice: mean $\pm$ sem=32.86 $\pm$ 2.075; *GlyT2::Cre; Cdh13^{fl/fl}* mice: mean $\pm$ sem=49.14 $\pm$ 3.744; Mann-Whitney U test: U=2.5, p=0.0029; duration (s): *Cdh13^{fl/fl}* mice: mean $\pm$ sem=29.02 $\pm$ 1.44; *GlyT2::Cre; Cdh13^{fl/fl}* mice: mean $\pm$ sem=51.48 $\pm$ 9.426; Mann-Whitney U test: U=8, p=0.0379) (Figure 30, B-D). However, when we split interaction type to nose-to-nose, nose-to-body and nose-to-anogenital contacts and measured frequency respectively, we found that control mice showed preference to nose-to-nose interact but *GlyT2::Cre; Cdh13^{fl/fl}* mice did not have preference among three types of contacts (Figure 30, C). This result is consistent with a previous study which reported that loss of Cdh13 does not contribute to sensory habituation in rat (King et al., 2017). Besides, we did not observe differences between control and *GlyT2::Cre; Cdh13^{fl/fl}* mice behaviors in the resident-intruder paradigm (Figure 30, E). Thus, no increase or decrease of aggression behavior were shown in *GlyT2::Cre; Cdh13^{fl/fl}* mice. Taken together, changes of *GlyT2::Cre; Cdh13^{fl/fl}* mice social interaction may reflect the impairment of social signal processing which result from loss of *Cdh13*.

Figure 30. *GlyT2-Cdh13*^{-/-} mice exhibit increase in reciprocal social interactions with loss of preference for contact region.

(A) Pattern of contacts during 10 minutes of reciprocal social interaction in a novel arena of same pair genotype: nose-nose, nose-body and nose-anogenital area. (B) During 10 minutes of social interaction, pairs of *GlyT2-Cdh13*^{-/-} mice display a higher preference to be in contact according to the result of total interaction duration ($p=0.002$, Mann-Whitney U-test) and took a shorter time to initiate the first contact ($p=0.0023$, Mann-Whitney U-test). (C) Evaluation of the number of contacts to nose, body and anogenital area shows a preference for contact to nose in pairs of control mice (one-way repeated measures ANOVA, area $F(1.676, 10.05)=16.42$; $p=0.0009$; individual $F(6, 12)=2.198$; $p=0.1157$; nose-nose vs. nose-body, $p=0.0101$; nose-nose vs. nose-anogenital, $p=0.0013$; nose-body vs. nose-anogenital, $p=0.6887$; Tukey's multiple comparisons test), but no preference for nose, body or anogenital in *GlyT2-Cdh13*^{-/-} mice (one-way repeated measures ANOVA, area $F(1.84, 11.04)=0.5055$; $p=0.6017$; individual $F(6, 12)=0.9288$; $p=0.5085$) despite an increase in number of total contacts (see Table 4). (D) Analysis of the first 3 of 10 minutes of social interaction revealed increased in the duration ($p=0.004$, Mann-Whitney U-test) and number of contacts ($p=0.03$, Mann-Whitney U-test) made by *GlyT2-Cdh13*^{-/-} mice. (E) In the resident-intruder paradigm to assess for inter-male aggression, no genotype differences was observed in latency to attack and duration of contact, including following and sniffing behavior. Data presented as mean $\pm$ SEM. Adult male mice, N=10-16/group. N, number of animals. See also Table 5.

Chapter 4. Discussion

This study explored the function of *Cdh13* in the cerebellum and how disruption of *Cdh13* perturbs animal behaviors. Conditionally knocking out *Cdh13* through *GlyT2::Cre*-mediated deletion resulted in decreased expression/location of GAD67 in the IGL of the cerebellar cortex, reduced inhibitory inputs to cerebellar Golgi cells and impairments of cognitive flexibility and social interaction behavior. These results provide evidence that *Cdh13* serves as a crucial link between the cerebellum and cognitive/social-related behaviors.

Analysis of the function of *Cdh13* via a conditional deletion strategy

To our knowledge, function of *Cdh13* has not been studied through a conditional knockout strategy in mice. Even though conventional knockout of *Cdh13* has been employed, the widespread knockout of *Cdh13* throughout the CNS makes it difficult to link the behavioral deficits with specific brain regions that lack *Cdh13* (Drgonova et al., 2016; Rivero et al., 2015). Our expression analysis is also the first to report the expression of *Cdh13* in the cerebellum. The conditional knockout of *Cdh13* with *GlyT2::Cre* mice provides an opportunity to understand the function of *Cdh13* in select regions of the brain at the molecular, electrophysiological and behavioral level. Selective ablation of this gene makes it possible to better understand the specific brain regions that are relevant for associated behavioral and electrophysiological deficits. Even though the number of regions where *Cdh13* was ablated in *GlyT2::Cre; Cdh13^{fl/fl}* mice was more than one, it is relatively low compared to *Cdh13* total knockout mice. The benefit of selective ablation of the gene here is two-fold: first, to find out which region is most related to the deficits, and second, to

understand the relevance of *Cdh13* within this region(s). Finally, regulatory sequences of *Cdh13* could be used to generate mouse genetics tools for targeting IGL inhibitory neurons.

There is lack of genetic tools for the monitor and manipulation of IGL inhibitory neurons including Golgi cells. Only a few Cre mouse lines have been reported to be used to achieve Cre-mediation recombination in IGL inhibitory neurons, including *mGluR2::Cre*, *Gad2::Cre* and *GlyT2::Cre* (Chen et al., 2011; Foster et al., 2015; Ishihara et al., 2010; Taniguchi et al., 2011). However, the recombination capability of both *GlyT2::Cre* and *Gad2::Cre* are not restricted to IGL inhibitory neurons (Ishihara et al., 2010; Taniguchi et al., 2011). According to our unpublished observations, *mGluR2::Cre* mice recombine in both excitatory and inhibitory neurons. In this thesis, we found that while *GlyT2::Cre* recombines in some Purkinje, stellate/basket cells, and a majority of IGL inhibitory neurons, IGL inhibitory neuron-specific manipulation was achieved because of the specific expression pattern of *Cdh13* in IGL inhibitory neurons. Therefore, instead of using *GlyT2* promoter, we propose the use of the *Cdh13* promoter as a promising candidate for studying the function of IGL inhibitory neurons in the future.

The expression of *Cdh13* in interneurons in the IGL of the cerebellum

From the seminal studies of Camillo Golgi and Ramon y Cajal, IGL inhibitory neurons have been shown to consist of a heterogeneous population (Cajal, 1888, 1889a, b; Golgi, 1885). Even though they share similarities such as large soma, complicated and widespread axonal plexus, dendrites that extend into the molecular layer, they also have many differences including the shape of soma, location of soma relative to PC, direction of dendritic plexus and length, thickness and arborization of axons (Cajal, 1888, 1889a, b; Golgi,

1885). Evidences from morphology, neurochemistry and electrophysiological studies support the conclusion that IGL inhibitory neurons are heterogeneous (Galliano et al., 2010; Geurts et al., 2003). For a period of time, the classification of all granular layer interneurons as Golgi cells were generally accepted before the identification of new types of IGL neurons. To date, IGL interneurons can be grouped into three major types of interneurons: Golgi cell, Lugaro cell and unipolar brush cell (Geurts et al., 2003).

Golgi cells and Lugaro cells are GABAergic and Glycinergic neurons, and unipolar brush cells are glutamatergic neurons (Dumoulin et al., 2001; Nunzi et al., 2001; Ottersen et al., 1987; Voogd and Glickstein, 1998). Some studies have proposed that Lugaro cells are mainly located close to the PCL, but others have shown that cells located in deep IGL also share characteristics with Lugaro cells (Laine and Axelrad, 1996, 2002). Hence, location cannot be the determining factor for distinguishing Golgi cells and Lugaro cells. On the other hand, the main method for distinguishing Lugaro cells from Golgi cells is based on their direct connection with Purkinje cells or the effect of serotonin (Dean et al., 2003). Lugaro cells receive inhibitory signals from Purkinje cells directly through chemical synapses, but no studies have been able to establish direct connection between Golgi cells and Purkinje cells (Altman and Bayer, 1997). Since *Cdh13* is expressed in GAD67-positive neurons in the IGL of the cerebellum, these GAD67-positive cells in the IGL could, in principle, be either Golgi cells or Lugaro cells. Therefore, our study has not resolved whether *Cdh13* is expressed in both types of IGL inhibitory neurons or in a subset. In the future, electrophysiological experiments will be needed to determine which subgroup of IGL inhibitory neuron *Cdh13* is expressed.

Roles of Cdh13 in mediating Golgi cell function

In our study, *Cdh13* was ablated in IGL inhibitory neurons and we show that *Cdh13* in around 90% of IGL inhibitory neurons was removed. The manipulation of *Cdh13* in IGL inhibitory neurons, including Golgi cells and Lugaro cells results in reduced inhibitory inputs of Golgi cells but did not affect excitatory inputs of Golgi cells. Together with unchanged amplitude, the reduction of frequency of IPSCs suggests that the decrease resulted from deficits in presynaptic neurons (Choi and Lovinger, 1997). The decrease in frequency could indicate a reduction of vesicle release, which suggests that the inhibitory vesicle pool at the presynaptic terminals are reduced (Sara et al., 2005). Another possibility is that there is a change in the number of synapses formed onto Golgi cells in the IGL. Inhibitory inputs onto Golgi cells consist of innervation from other Golgi cells, Lugaro cells and nucleocortical fibers that originate from inhibitory neurons in the deep cerebellar nuclei (Ankri et al., 2015; Dumoulin et al., 2001; Hull and Regehr, 2012). The change of synaptic number may be a result of disruption of Cdh13 function during development (e.g. axon guidance or synaptic development). The disruption of the inhibitory inputs to Golgi cells indicates an interruption of single or multiple types of these presynaptic terminals.

A previous *Cdh13* knockout study shows that the knockout of *Cdh13* at presynaptic cells results in increased mIPSC frequency in the hippocampus (Rivero et al., 2015). In our analysis, *Cdh13* is ablated at most of the postsynaptic cells (i.e. Golgi cells) and removed partially at presynaptic cells (i.e. Golgi/Lugaro cells). Different from the study in the hippocampus (Rivero et al., 2015), we observed a reduction of the frequency of mIPSC in Golgi cells of *GlyT2::Cre; Cdh13^{fl/fl}* mice. While the difference between our result and previous study may be caused by distinctions between the connectivity within the hippocampus and the cerebellum, it could also be caused by specificity of activities of Cdh13

involved in synaptic functions. Both homophilic and heterophilic interaction between cadherins has been shown contribute to cell-cell adhesion (Hirano and Takeichi, 2012). As a unique interface between two neurons, the formation and maintenance of synapses also need adhesion molecules, and members of cadherin superfamily may form homodimers or heterodimers or both at synaptic regions in the nervous system (Seong et al., 2015).

In the previous study examining the role of Cdh13 in the hippocampus (Rivero et al., 2015), Cdh13 is only expressed in presynaptic neurons so the ablation of *Cdh13*, in principle, results in the disruption of heterophilic interaction between Cdh13 and other proteins at or around the postsynaptic region (Rivero et al., 2015). In our study, *Cdh13* in both pre- and postsynaptic cells was ablated. Even though *Cdh13* was likely deleted only in a subset of presynaptic cells since Golgi cell inhibitory inputs comes from additional cell types including Lugaro cells and nucleocortical inhibitory neurons, this deletion possibly disrupts the homophilic interaction between Cdh13 at or around synapse. The importance of homophilic interaction between Cdh13 has been shown in studies of axon guidance and neurite outgrowth (Ciatto et al., 2010; Hayano et al., 2014; Vestal and Ranscht, 1992). The disruption of inhibitory input caused by heterophilic binding in our *GlyT2::Cre; Cdh13^{fl/fl}* tissue also remains a possibility. Cdh13 at or around the postsynaptic neuron can interact with proteins localized on the presynaptic neuron and vice versa. On postsynaptic compartments, GABA_A receptor subunit $\alpha 1$ is a candidate of Cdh13 target on the postsynaptic site, and integrin subunit $\beta 1$ could be a candidate of Cdh13 target on the presynaptic site (Mukoyama et al., 2007; Rivero et al., 2013). Altogether, future identification of the binding partners of Cdh13 in contextually relevant regions of the nervous system will provide a better understanding of the underlying mechanisms of Cdh13-mediated synaptic functions.

Cdh13 may also be involved in mediating gap junctions between Golgi cells. Golgi cells have been reported to connect with other Golgi cells by using gap junction even before their chemical connections were reported (Dugue et al., 2009). The gap junctions of Golgi cells play a role in regulating Golgi cell synchronized or desynchronized firing depending on inputs from mossy fiber terminals and has been considered as an important determinant of low-frequency oscillation in the IGL of the cerebellum (Robinson et al., 2017; Vervaeke et al., 2010). Interestingly, proteins used to form gap junctions (connexin proteins) have been reported to interact with members in cadherin superfamily or proteins bind with cadherins (Wei et al., 2004). This suggests *Cdh13* ablation in Golgi cell could disrupt gap junctions. Future Golgi cell gap junction analysis can be done on *GlyT2::Cre; Cdh13^{fl/fl}* mice. First, the type and amount of proteins such as connexin 36, which make up gap junction among Golgi cells can be compared between Golgi cells of *Cdh13^{fl/fl}* and *GlyT2::Cre; Cdh13^{fl/fl}* mice by immunohistochemistry (Szoboszlay et al., 2016). Second, difference in electrophysiological properties of gap junctions between Golgi cells of *Cdh13^{fl/fl}* and *GlyT2::Cre; Cdh13^{fl/fl}* mice can be assessed. Finally, stimulus-induced gap junction mediated inhibition can be tested in Golgi cell and granule cells and from which models of IGL loops can be formulated. By using these methods, we hope to gain more insight into the cellular mechanisms underlying the contribution of Golgi cells to IGL activity and cerebellar networks.

Changes in the synaptic organization of the cerebellum may account for behavioral deficits of *GlyT2::Cre; Cdh13^{fl/fl}* mice

The cerebellum receives inputs from other parts of the brain mainly through climbing fibers and mossy fibers (Sillitoe and Joyner, 2007). Mossy fibers project to the IGL, or the input layer of the cerebellum and activate granule cells directly or inhibit granule cells through activating Golgi cells (feedforward inhibition) (Geurts et al., 2003). Golgi cells could

inhibit granule cells by using inhibitory synapses formed on granule cells or by maintaining GABA level at a certain concentration which can activate high-affinity GABAA receptors at the extrasynaptic regions of granule cells (tonic inhibition) (Geurts et al., 2003). Granule cells could regulate Purkinje cells and therefore, make influences on the outputs of the cerebellum (Geurts et al., 2003). In our *GlyT2::Cre; Cdh13^{fl/fl}* mice, the amount of GAD67, the enzyme critical for GABA synthesis reduced in the IGL may result in the general reduction of GABA. The decrease of GABA in the IGL may cause the disruption of tonic inhibition of granule cells in the cerebellar input layer. Tonic inhibition has been reported to provide majority inhibitory charge of granule cells and the manipulation of tonic inhibition on granule cells affect information process (Duguid et al., 2012; Hamann et al., 2002). Hence, the behavioral deficits of *GlyT2::Cre; Cdh13^{fl/fl}* mice could be accounted for by the decrease of GAD67 in the IGL. Moreover, the impairments of cognitive flexibility and social function may also be caused by reduced sIPSC frequency recorded on Golgi cells. To receive and filter a variety of mossy fiber inputs accurately, the temporal control of Golgi cell spontaneous activity and mossy fiber is required (Kanichay and Silver, 2008). The disruption of Golgi cell sIPSC may result in the change of spontaneous spikes, which further alters the consequence of firing rate controlled by mossy fiber inputs. As a target of Golgi cells, granule cell activity could be disturbed because of impaired feedforward inhibition. In normal mice, granule cell firing could encode predictive signals for both motor learning and cognitive-related process such as reward expectation (Giovannucci et al., 2017; Wagner et al., 2017). Failure of precisely generating predictive signal in granule cells may lead to unexpected behavioral abnormalities.

A link between cerebellar network with cognitive and social behaviors

The cerebellum has been reported to link to executive function and social-related behavior (Schmahmann, 2010). However, limited evidences have been shown in rodents. A recent study linked granule cells to reward expectation by using calcium imaging (Mark J. Wagnern 2017). Results of manipulation of Purkinje cells also associate the change of the cerebellum with social and cognitive-related behavioral deficits: knockout of *Tsc1* in Purkinje cell cause ASD-like social behavior impairments, cognitive inflexibility and vocalization deficiency (Tsai et al., 2012). Similar ASD related behavioral deficits also been found in mice of which *SHANK2* or *PTEN* was ablated specifically in Purkinje cells (Cupolillo et al., 2016; Peter et al., 2016). Furthermore, the cognitive and social behavioral deficits and functional disruption of the cerebellum can also be observed in ASD models such as *IB2*, *CAPS2*, *Mecp2*, *Cntnap2*, *Engrailed-2*, *patDp*, *Fragile X* and *neuroligin-3* (Kloth et al., 2015; Wang et al., 2014). Here, we reported another cerebellar disruption-related mouse model for studying cognitive and social behavior: *GlyT2::Cre; Cdh13^{fl/fl}*. Instead of examining the Purkinje cells, we mainly focused our studies on IGL inhibitory neurons.

Additional studies must be done on Golgi cells or other IGL inhibitory neurons in order to better understand the contributions of IGL inhibitory neurons to cognitive and social information processing. An important analysis to study cognitive function-related contribution of Golgi cells, will be recording the firing pattern of Golgi cells and the association cortex simultaneously. Reported synchrony of Golgi cell firing with slow oscillation of the primary somatosensory barrel cortex suggests a functional relationship between these two regions (Ros et al., 2009). Similar approach to record the activities of cognitive behavior-related cortical and cerebellar regions can also be performed in future studies. Furthermore, similar to cognitive-related studies on granule cells (Mark J. Wagnern

2017), calcium imaging can also be used to study the activities of IGL inhibitory neurons by using *Cdh13* promotor to target the expression of GCaMP, an indicator of calcium, in IGL (Giovannucci et al., 2017).

The deficit in cognitive flexibility of *GlyT2::Cre; Cdh13^{fl/fl}* mice are exacerbated by overtraining phase

From our results, we observed impairments in cognitive flexibility of *GlyT2::Cre; Cdh13^{fl/fl}* mice compared to control mice, especially in the first phase after overtraining. In both control and *GlyT2::Cre; Cdh13^{fl/fl}* mice, overtraining phase was included. In our experiments, the phases IDS1 and IDS1R consisted of basically a group of repeated phases of CD and CDR with different materials. After IDS1 and IDS1R, another group of repeated phases, IDS2 and IDS2R were given. Even though there was no overtraining from CDR to IDS1, however, overtraining did take place from IDS1R to IDS2. The performance of *GlyT2::Cre; Cdh13^{fl/fl}* mice was indistinguishable from control mice from CDR to IDS1, but declined from IDS1R to IDS2 compared to control. Since overtraining served as a variable in this experiment, we interpreted that the overtraining contributed to the differences we observed between *Cdh13* mutant and control mice.

It remains probable that timing could influence the way mice behaves in a cognitive flexibility test. Since CD, CDR, IDS1 and IDS1R were earlier phases compared to IDS2 and IDS2R, overtraining might not necessarily represent the only variable introduced because animals may behave differently after being given different amount of training. However, we reasoned that if the overtraining did not contribute to cognitive flexibility deficits of *GlyT2-Cdh13^{-/-}* mice, the mice should have performed better from IDS1R to IDS2 according to an idea known as

overtraining reversal effect (ORE), which proposed that overtraining could facilitate reversal learning because the mice become familiar to the rules (Van Golf Racht-Delatour and Massiou, 2000). We did not observe an overtraining reversal effect in *GlyT2-Cdh13*^{-/-} mice. Instead, the *GlyT2-Cdh13*^{-/-} mice performed worse after overtraining. Thus, our observations suggest that overtraining intensifies any potential disruptions in cognitive flexibility due to deletion of *Cdh13*.

Comparison of the results of two-choice digging task between *Cdh13* conditional mutant mice and lurcher mice

The functional relationship between the cerebellum and cognitive flexibility has been tested in rodents (Dickson et al., 2017; Dickson et al., 2010). In this study, lurcher mice, which have significant loss of Purkinje cells, granule cells and neurons in the inferior olive, were trained to associate visual stimuli to reward with the response of touching screen for selection (Dickson et al., 2017; Dickson et al., 2010; Vogel et al., 2007). Various visual stimuli, lines and shapes were used as two dimensions (Dickson et al., 2017). This study differs from our test setup in that extra-dimensional stages were employed (Dickson et al., 2017). From simple discrimination to intra-dimensional discrimination shift phases, lines were used as the relevant dimension and shapes were used as the irrelevant dimension (Dickson et al., 2017). When the extra-dimensional stages commenced, shapes became relevant dimension and lines were considered as irrelevant dimension. In our test, bowl covers were always represented the relevant dimension. Additionally, the arrangements of discrimination phases are also different between our study and those by others. In the study of lurcher mice, no reversal phases were carried out after CD phase and four consecutive IDS phases were performed before an IDSR phase (Dickson et al., 2017). In our study, three reversal phases were presented after CD phase, the first IDS phase and the second IDS phase

respectively (Dickson et al., 2017). In result, the lurcher mice showed an impairment of cognitive flexibility at the IDSR phase (after 4 intra-dimensional discrimination) but the cognitive flexibility deficits was not obvious in our *GlyT2::Cre; Cdh13^{fl/fl}* mice in the first and second IDSR phases and the deficits were obvious only during the first discrimination phase after overtraining.

Although protocols and results in two studies are different, similar cognitive flexibility impairments were observed in both studies (during reversal phases or after overtraining). The cognitive flexibility deficits were not obvious during reversal discrimination phases in our study because of, perhaps, the quick shifting from discrimination phases to discrimination reversal phases. In the analysis of lurcher mice, four discrimination phases were presented before the discrimination reversal phase (Dickson et al., 2017). In our test, only one discrimination phase was given before each discrimination reversal phase. Quickly shifting between discrimination and discrimination reversal phases may result in incomplete learning consolidation, which may reduce the inhibitory effect of previous discrimination phase to discrimination reversal phase allowing mice to shift effectively between different rules. Importantly, the genetic manipulation in our study is more subtle than the significant loss of many important classes of cerebellar neurons in the lurcher mice. This distinction might explain why the reversal learning deficits were not obvious without strengthening by overtraining.

The cerebellum and cognitive flexibility, reversal learning and attentional set shifting

Cognitive flexibility is a complex mental process which allows animal to adjust their behaviors properly based on changes in the environment (Dajani and Uddin, 2015). Effective

performance of cognitive flexibility requires normal processing of cognitive functions such as attention, learning and working memory (Logue and Gould, 2014). In different tests carried out in laboratories, various aspects of cognitive flexibility can be assessed. In rodents, reversal learning task and attentional set shifting task are two important paradigms (Chuang, 2014; Garner et al., 2006). The two-choice digging task used in our lab is a type of reversal learning tasks, which encompasses a series of discriminations without changing dimension of stimulus (Chuang, 2014). To shift within the same dimension, sustained attention is required. Different from reversal learning task, attentional set shifting task contains shifting between dimensions (i.e. shifting between intra-dimensional and extra-dimensional discrimination) (Logue and Gould, 2014). To shift between dimensions, the switch of selective attention is required. Thus, the brain regions required for carrying out reversal learning and attentional set shifting are likely different.

In addition to the cerebellum, other regions in the brain such as the agranular insular cortex (AI), the orbital frontal cortex (OFC) and the striatum have also been shown contribute to reversal learning (Bissonette et al., 2008; Clarke et al., 2008; Hamilton and Brigman, 2015). For attentional set shifting, the dorsolateral prefrontal cortex (DLPFC) and the medial prefrontal cortex (mPFC) are mainly involved (Bissonette et al., 2008; Dias et al., 1997). Although no anatomical linkage between the cerebellum and the OFC has been described to date, tracing results showed that the cerebellum forms a close loop with the DLPFC (Buckner et al., 2011; Kelly and Strick, 2003; Schmahmann and Pandya, 1997). Moreover, functional connectivity studies have reported connectivity between the cerebellum and the mPFC, and between the cerebellum and the anterior insular cortex (Buckner et al., 2011; Dobromyslin et al., 2012). Interestingly, cognitive flexibility deficits have been reported in animals with disruptions in the DLPFC or mPFC through assessment of attentional set-shifting task but not

reversal learning task (Bissonette et al., 2008; Dias et al., 1997). However, OFC lesion does not affect the performance of animals in set-shifting tasks when shift from intra-dimension to extra-dimension phases (Bissonette et al., 2008). The connection between the cerebellum and the DLPFC or mPFC suggests possible involvement of the cerebellum in the DLPFC- or mPFC-mediated cognitive functions. Together with previous result shown in the *lurcher* mice, our findings support the possible contribution of the cerebellum to the OFC-related cognitive flexibility. Therefore, we propose that the cerebellum is a critical component of the OFC, DLPFC and mPFC network.

Loss of nose-to-nose preference in *Cdh13* conditional mutant mice links *Cdh13*-ablated brain regions with social network

To assess the reciprocal social interaction between *GlyT2::Cre; Cdh13^{fl/fl}* mice, we analyzed the nose-to-nose, nose-to-body and nose-to-anogenital respectively. Control mice prefer nose-to-nose contact over the other two types of contact whereas this preference is lost in *GlyT2::Cre; Cdh13^{fl/fl}* mice (Figure 30, C). Analysis of the social behavior of an autism mouse model, BTBR mice, revealed that the duration of face sniffing during adult reciprocal social interaction test is reduced, but the duration of anogenital sniffing remains the same as control mice (McFarlane et al., 2008). Loss of preference during reciprocal social interaction may represent a form of social behavioral deficits. The phenotype might also be compared to eye-contact avoidance commonly reported in autism patients (Madipakkam et al., 2017). Furthermore, *in vivo* recordings provide evidence that the signals transferred through facial/nose area interaction use a different pathway from information sent from anogenital area interaction (Luo et al., 2003). By recording from the accessory olfactory bulb, a region innervated by vomeronasal organs, a previous study shows that the frequency of neuronal

firing is highly increased by nose/facial contact but not anogenital contact (Luo et al., 2003). Therefore, the phenotype of the loss of nose-to-nose preference in *Cdh13* conditional mutant mice suggests that *Cdh13* function might be specific to a pathway specific to nose/facial detection.

Results in our studies indicate that *Cdh13* ablation leads to social behavioral impairment, but the exact regions in the brain where the deletion of *Cdh13* contributes to this defect is still unclear. In *GlyT2::Cre; Cdh13^{fl/fl}* mice, we show that *Cdh13* was deleted in several regions including the cerebellum and piriform cortex. Even though studies have shown that the cerebellum may be involved in social function, the piriform cortex has also been demonstrated to play an important role in odor recognition memory and processing, a critical process for social behaviors (Bekkers and Suzuki, 2013; Savic et al., 2000; Schmahmann, 2010; Stettler and Axel, 2009). Therefore, the social deficits we reported in *GlyT2::Cre; Cdh13^{fl/fl}* mice could be consequences of *Cdh13* deletion in the cerebellum, or in the piriform cortex or in both regions. Results from previous studies support a role for both the cerebellum and the piriform cortex in social behaviors and social cue processing of rodents (Choe et al., 2015; Tsai et al., 2012). Moreover, studies have linked both the cerebellum and the piriform cortex to epilepsy, suggesting functional connectivity between these two regions (Vaughan and Jackson, 2014).

In addition to the cerebellum and the piriform cortex, the contribution of other brain regions to social interaction has also been reported including amygdala, hippocampus, OFC and raphe nuclei (File and Seth, 2003). Anatomical or functional connectivity of the cerebellum with these regions has been reported (Magal and Mintz, 2014; Pierce et al., 1977; Sokolov et al., 2017). The connection suggests that the cerebellum may work together with

these regions to integrate the social function and that *Cdh13* may play a critical role in this process.

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Appendix

Table 4. Analysis of the number of *Cdh13*⁺ cells in different brain regions of *GlyT2-Cdh13*^{-/-} mice

	Control	<i>GlyT2-Cdh13</i> ^{-/-}		
Cerebellum (#/ μm^2)	1.98x10 ⁻⁵ ⁵ ±1.42x10 ⁻⁵ 6(N=3; n=16)	2.17x10 ⁻⁶ ±3.48x10 ⁻⁷ (N=3; n=16)	U=0; p<0.0001	Mann-Whitney U
Pir (#/ μm^2)	2.2x10 ⁻⁴ ±3.35x10 ⁻⁵ ⁵ (N=3; n=27)	1.26x10 ⁻⁴ ±1.79x10 ⁻⁵ (N=3; n=29)	U=253; p=0.0228	Mann-Whitney U
OFC (#/ μm^2)	1.81x10 ⁻⁵ ⁴ ±1.87x10 ⁻⁵ (N=3; n=18)	1.79x10 ⁻⁴ ±1.78x10 ⁻⁵ (N=3; n=16)	U=142; p=0.9593	Mann-Whitney U
Ins (#/ μm^2)	5.11x10 ⁻⁵ ⁴ ±3.15x10 ⁻⁵ (N=3; n=17)	4.57x10 ⁻⁴ ±3.44x10 ⁻⁵ (N=3; n=18)	U=118; p=0.2584	Mann-Whitney U
Rhi (#/ μm^2)	5.21x10 ⁻⁵ ⁴ ±4.17x10 ⁻⁵ (N=3; n=14)	4.42x10 ⁻⁴ ±3.53x10 ⁻⁵ (N=3; n=13)	U=58; p=0.1159	Mann-Whitney U
Hypothalamus (#/ μm^2)	3.13x10 ⁻⁵ ⁴ ±5.11x10 ⁻⁵ (N=3; n=12)	2.91x10 ⁻⁴ ±5.54x10 ⁻⁵ (N=3; n=12)	U=69; p=0.8874	Mann-Whitney U
DR (#/ μm^2)	1.87x10 ⁻⁵ ⁴ ±1.07x10 ⁻⁵ (N=3; n=6)	1.82x10 ⁻⁴ ±2.62x10 ⁻⁵ (N=3; n=6)	U=14; p=0.5887	Mann-Whitney U

Table 5. Analysis of the expression of GAD67 in the IGL of the *GlyT2-Cdh13*^{-/-} mice

	Control	<i>GlyT2-Cdh13</i> ^{-/-}		
GAD67 intensity				
Crus2 (fl.u)	19.04±1.14 (N=3; n=22)	13.7±0.99 (N=3; n=20)	U=99; p=0.0019	Mann-Whitney U
lobule IX (fl.u)	17.55±1.08(N=3; n=22)	13.2±0.72(N=3; n=24)	U=103; p=0.0003	Mann-Whitney U

Table 6. Analysis of the electrophysiological properties of Golgi cells lacking *Cdh13*

	Control	<i>GlyT2-Cdh13</i>^{-/-}		
sIPSC frequency (Hz)	2.25±0.23 (N=9; n=12)	1.25±0.21 (N=8; n=14)	U=31; p=0.0054	Mann-Whitney U
mIPSC frequency (Hz)	1.89±0.22 (N=9; n=12)	0.89±0.14 (N=8; n=14)	U=26; p=0.002	Mann-Whitney U
sIPSC amplitude (pA)	23.72±2.79 (N=9; n=12)	22.92±1.62 (N=8; n=14)	U=71; p=0.5267	Mann-Whitney U
mIPSC amplitude (pA)	22.35±2.23 (N=9; n=12)	22.14±1.6 (N=8; n=14)	U=83; p=0.9798	Mann-Whitney U
sEPSC frequency (Hz)	3.29±0.37 (N=8; n=10)	3.33±0.32 (N=7; n=11)	U=52; p=0.8633	Mann-Whitney U
mEPSC frequency (Hz)	2.54±0.32 (N=8; n=10)	2.61±0.32 (N=7; n=11)	U=54; p=0.9725	Mann-Whitney U
sEPSC amplitude (pA)	19.79±0.75 (N=8; n=10)	20.33±0.78 (N=7; n=11)	U=46; p=0.5573	Mann-Whitney U
mEPSC amplitude (pA)	18.45±0.98 (N=8; n=10)	18.62±0.88 (N=7; n=11)	U=52; p=0.8633	Mann-Whitney U

Table 7. Analysis of the consequences of *Cdh13* deletion in Golgi cells on general motor behaviors

	Control	<i>GlyT2-Cdh13</i>^{-/-}		
Rotarod				
(Latency to fall (s))	N=10	N=16		
trial 1	57.74±5.01	58.94±4.06	Interaction F(5,120)=0.8819; p=0.4956	2-way repeated measures ANOVA
trial 2	56.03±4.84	59.99±6.26	Genotype F(1,24)=0.5444; p=0.4678	
trial 3	82.49±12.55	73±5.57	Trial F(5,120)=5.794; p<0.0001	
trial 4	64.9±11.47	62.62±5.29		
trial 5	73.33±7.47	72.41±6.01		
trial 6	93.89±12.34	74.83±5.29		
Gait	N=5; n=35	N=5; n=34		
Stride length (cm)	7.51±1.41	6.86±0.16	U=440; p=0.0631	Mann- Whitney U
Open field	N=12	N=19		
Distance (cm)	3549±334.9	3674±234.7	U=100; p=0.578	Mann- Whitney U
Velocity (cm/s)	8.47±0.79	8.79±0.56	U=9; p=0.551	Mann- Whitney U
Center visits	11.75±1.26	14.95±1.67	U=83; p=0.214	Mann- Whitney U
Stay duration center zone (s)	15.88±3.88	20.01±2.69	Interaction F(1,58)=1.101; p=0.2985	2-way ANOVA
Stay duration corner zone (s)	88.56±16.18	75.72±7	Genotype F(1,58)=0.29; p=0.5921; Zone F(1,58)=63.01; p<0.0001	
Marble burying and digging	N=10	N=16		
Number of buried marbles	1.4±0.22	2.38±0.52	U=63; p=0.3095	Mann- Whitney U

Table 8. Analysis of consequences of *Cdh13* deletion in Golgi cells on cognitive flexibility

	Control	<i>GlyT2-Cdh13</i> ^{-/-}		
2-choice digging task	N=6	N=11		
Trials to criterion across stages				
SD (#)	20±2.29	25.73±4.97	Interaction F(6, 90)=0.6298; p=0.706	2-way repeated measures ANOVA
CD (#)	47.67±13.94	38.91±5.96	Genotype F(1, 15)=4.766; p=0.0453	
CD R (#)	42.83±12.16	60.82±11.97	Task stage F(6, 90)=3.005; p=0.0101	
IDS1 (#)	49.33±13.71	45.64±6.26		
IDS1 R (#)	38.83±5.78	43±4.27		
IDS2 (#)	39.17±6.35	63.36±7.49		
IDS2 R (#)	58.33±22.47	74.91±12.98		
Trials before overtraining (#)	199.7±14.57	214.1±12.64	Interaction F(1, 15)=0.5237; p=0.4804	2-way repeated measures ANOVA
Trials after overtraining (#)	97.5±22.84	138.3±12.65	Genotype F(1, 15)=23.90; p=0.045; OVT F(1,15)=4,766; p=0.0002	
Error before overtraining (#)	16.6±1.47		p=0.69	Wilcoxon test
Error after overtraining (#)	21.67±6.64			
		17.8±1.42	p=0.0059	Wilcoxon test
		32.09±3.57		
Error at IDS 2 (#)	17.5±3.19	28.82±3.57	U=10.50; p=0.0218	Mann-Whitney U

Table 9. Analysis of consequences of *Cdh13* deletion in Golgi cells on social related behaviors-1

	Control	<i>GlyT2-Cdh13</i> ^{-/-}		
Olfaction test	N=8	N=14		
Water 1 (sniffing time (s))	6.75±1.26	11.01±4.7	Interaction F(11,220)=0.26; p=0.99	2-way repeated measures ANOVA
Water 2 (sniffing time (s))	1.78±0.39	3.26±1.09	Genotype F(1,20)=0.475; p=0.5	
Water 3 (sniffing time (s))	4.37±2.65	4.18±2.02	Odor F(11,220)=19.74; p<0.0001	
Rose 1 (sniffing time (s))	11.18±3.16	16.14±3.8		
Rose 2 (sniffing time (s))	1.48±0.32	4.67±1.8		
Rose 3 (sniffing time (s))	2.48±1.56	1.67±0.72		
Social 1a (sniffing time (s))	55.58±10.06	58.64±8.5		
Social 2a (sniffing time (s))	24.66±9.3	29.53±8.64		
Social 3a (sniffing time (s))	9.79±3.7	18.39±6.49		
Social 1b (sniffing time (s))	21.02±7.8	24.69±5.05		
Social 2b (sniffing time (s))	11.02±5.5	5.23±0.9		
Social 3b (sniffing time (s))	8.91±7.1	10.29±3.03		

Table 10. Analysis of consequences of *Cdh13* deletion in Golgi cells on social related behaviors-2

	Control	<i>GlyT2-Cdh13</i> ^{-/-}		
Reciprocal social interaction	N=7	N=7		
Duration (10 mins) (s)	48.39±3.07	84.54±7.75	U=2.00; p=0.002	Mann-Whitney U
Latency to first contact (s)	11.92±2.66	2.06±0.56	U=2.00; p=0.0023	Mann-Whitney U
# of contacts in 10 mins	N=7	N=7		
Nose to nose (#)	19.14±1.44	23.43±2.33	Control: Area F(1.676,10.05)=16.42; p=0.0009; Individual F(6,12)=2.198; p=0.1157	One-way repeated measures ANOVA
Nose to body (#)	12.29±1.27	21.43±1.66	<i>GlyT2-Cdh13</i> ^{-/-} : Area F(1.84,11.04)=0.5055; p=0.6017; Individual F(6,12)=0.9288; p=0.5085	
Nose to anogenital (#)	10.71±1.21	20.57±2.07	Control: Nose to nose vs. nose to body: p=0.0101; Nose to nose vs. nose to anogenital: p=0.0013; Nose to body vs. nose to anogenital: p=0.6887	Tukey's multiple comparisons test
Total (#)	42.14±2.84	65.43±3.44	U=1.00; p=0.0012	Mann-Whitney U
Duration (3 mins) (s)	19.37±2.01	33.06±2.69	U=3.00; p=0.0041	Mann-Whitney U
Contacts (3 mins) (#)	9.29±0.92	16.29±2.01	U=8.00; p=0.0332	Mann-Whitney U
Resident/intruder	N=10	N=16		
Attack latency (s)	457.7±56.41	526.8±32.96	U=58.00; p=0.182	Mann-Whitney U
Contact duration (first 2 mins) (s)	21.34±3.34	21.57±2.89	U=57.00; p=0.693	Mann-Whitney U