Research Articles: Cellular/Molecular

Hyper-excitability and hyper-plasticity disrupt cerebellar signal transfer in the IB2 KO mouse model of autism

Teresa Soda¹,², Lisa Mapelli¹, Francesca Locatelli¹, Laura Botta³, Mitchell Goldfarb⁴, Francesca Prestori¹ and Egidio D’Angelo¹,⁵

¹Dept of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy
²Museo Storico della Fisica e Centro Studi e Ricerche Enrico Fermi, Rome, Italy
³Dept of Biology and Biotechnology “L. Spallanzani”, University of Pavia, Pavia, Italy
⁴Dept of Biological Sciences, Hunter College, New York, USA
⁵Brain Connectivity Center, C. Mondino National Neurological Institute, Pavia, Italy

https://doi.org/10.1523/JNEUROSCI.1985-18.2019

Received: 27 July 2018
Revised: 22 December 2018
Accepted: 8 January 2019
Published: 29 January 2019

Author contributions: T.S., L.M., L.B., and F.P. performed research; T.S., L.M., and F.P. analyzed data; L.M., F.P., and E.U.D. wrote the paper; M.G. and E.U.D. designed research; M.G. contributed unpublished reagents/analytic tools.

Conflict of Interest: The authors declare no competing financial interests.

This project has received funding from: the European Union’s Horizon 2020 Framework Program for Research and Innovation under Grant Agreement No. 720270 (Human Brain Project SGA1); European Union grant Human Brain Project (HBP-604102) to ED; Fermi grant CNL to ED; Blue-Sky Research grant of the University of Pavia (BSR77992) to LM., Particular acknowledgments are made to Simona Tritto for technical assistance.

Correspondence should be addressed to: Prof. Egidio D’Angelo, University of Pavia, Dept of Brain and Behavioral Sciences, via Forlanini 6, 27100 Pavia, Italy, dangelo@unipv.it

Cite as: J. Neurosci 2019; 10.1523/JNEUROSCI.1985-18.2019

Alerts: Sign up at www.jneurosci.org/alerts to receive customized email alerts when the fully formatted version of this article is published.
Hyper-excitability and hyper-plasticity disrupt cerebellar signal transfer in the IB2 KO mouse model of autism

Teresa Soda1,2**, Lisa Mapelli1**, Francesca Locatelli1, Laura Botta1, Mitchell Goldfarb4, Francesca Prestori1*, Egidio D’Angelo1,5*

1 Dept of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy
2 Museo Storico della Fisica e Centro Studi e Ricerche Enrico Fermi, Rome, Italy
3 Dept of Biology and Biotechnology ”L. Spallanzani”, University of Pavia, Pavia, Italy
4 Dept of Biological Sciences, Hunter College, New York, USA
5 Brain Connectivity Center, C. Mondino National Neurological Institute, Pavia, Italy

* co-last authors
** co-first authors

Correspondence should be addressed to:
Prof. Egidio D’Angelo
University of Pavia
Dept of Brain and Behavioral Sciences
via Forlanini 6, 27100 Pavia, Italy
dangelo@unipv.it

Number of pages: 37
Number of tables: 1
Number of figures: 9
Number of words: 161-Abstract, 646-Introduction, 1489-Discussion
Acknowledgments. This project has received funding from: the European Union’s Horizon 2020 Framework Program for Research and Innovation under Grant Agreement No. 720270 (Human Brain Project SGA1); European Union grant Human Brain Project (HBP-604102) to ED; Fermi grant CNL to ED; Blue-Sky Research grant of the University of Pavia (BSR77992) to LM. Particular acknowledgments are made to Simona Tritto for technical assistance.

Author contribution. TS performed the bulk of electrophysiological experiments and data analysis, LM performed the whole set of imaging experiments and data analysis, FL performed part of the electrophysiological experiments, LB performed mice genotyping, MG provided the mice and contributed to paper discussion, FP performed the initial experiments and data analysis, ED LM and FP wrote the paper and ED coordinated the work.

Abstract

Autism spectrum disorders (ASD) are pervasive neurodevelopmental conditions that often involve mutations affecting synaptic mechanisms. Recently, the involvement of cerebellum in ASD has been suggested but the underlying functional alterations remained obscure. We investigated single-neuron and microcircuit properties in IB2 KO mice of either sex. The IB2 gene (chr22q13.3 terminal region) deletion occurs in virtually all cases of Phelan–McDermid syndrome, causing autistic symptoms and a severe delay in motor skill acquisition. IB2 KO granule cells showed a larger NMDA receptor-mediated current and enhanced intrinsic excitability raising the excitatory/inhibitory balance. Furthermore, the spatial organization of granular layer responses to mossy fibers shifted from a Mexican hat to stovepipe hat profile, with stronger excitation in the core
and weaker inhibition in the surround. Finally, the size and extension of long-term synaptic plasticity was remarkably increased. These results show for the first time that hyper-excitability and hyper-plasticity disrupt signal transfer in the granular layer of IB2 KO mice supporting cerebellar involvement in the pathogenesis of ASD.

Significance statement

This paper shows for the first time a complex set of alterations in the cerebellum granular layer of a mouse model (IB2 KO) of autism spectrum disorders. The IB2 KO in mice mimics the deletion of the corresponding gene in the Phelan McDermid syndrome in humans. The changes reported here are centered on NMDA receptor hyper-activity, hyper-plasticity and hyper-excitability. These, in turn, increase the excitatory/inhibitory balance and alter the shape of center/surround structures that emerge in the granular layer in response to mossy fiber activity. These results support recent theories suggesting the involvement of cerebellum in autism spectrum disorders.

Introduction

Autism Spectrum Disorders (ASDs) are pervasive developmental disorders characterized by impairment in social communication and social interaction and by the presence of repetitive behaviors and/or restricted interests. ASDs cover a spectrum of different clinical conditions ranging from severely hypofunctional to hyperfunctional, and show abnormalities in different brain regions. Although most attention has been given so far to the cerebral cortex, increasing evidence implicates also the cerebellum (Amaral, 2011; Betancur, 2011; Ellegood et al., 2015). Cerebellar lesions often cause autistic-like symptoms (Hampson and Blatt, 2015) and perinatal cerebellar injuries are the greatest non-genetic risk factor for ASD (Bolduc and Limperopoulos, 2009; Limperopoulos et al., 2009; Bolduc et al., 2011; Wang et al., 2014; Mosconi et al., 2015). Moreover, cerebellar alterations
are found in several syndromic forms of ASD, like Phelan-McDermid, Fragile X, Tuberous Sclerosis and Rett syndrome [for recent reviews, see (Courchesne and Allen, 1997; Schmahmann, 2004; Allen, 2006; Ito, 2008; D'Angelo and Casali, 2013; Broussard, 2014; Hampson and Blatt, 2015; Mosconi et al., 2015; Zeidán-Chuliá et al., 2016)]. This raises a main question: are there any alterations of cerebellar microcircuit functions in ASD?

ASDs are often associated with mutations in genes coding for synaptic proteins (Qiu et al., 2012; Banerjee et al., 2014; De Rubeis and Buxbaum, 2015; Kim et al., 2016) bringing about neurotransmission abnormalities (Curatolo et al., 2014; Ellegood et al., 2015; Kloth et al., 2015; Mercer et al., 2016; Sztainberg and Zoghbi, 2016; Tsai, 2016; Tu et al., 2017). The consequent microcircuit alterations have mainly been analyzed in the neocortex revealing that: (i) hyper-reactivity to stimulation, accompanied by altered neuronal excitability and synaptic plasticity, was related to increased glutamatergic transmission (Rinaldi et al., 2007; Markram et al., 2008; Rinaldi et al., 2008b; Markram and Markram, 2010); (ii) dysregulation of the excitatory/inhibitory (E/I) balance was related to various alterations at excitatory and inhibitory synapses (Rubenstein and Merzenich, 2003; Gogolla et al., 2009; Uzunova et al., 2015); (iii) altered modular organization of microcircuits (Casanova, 2003, 2006; Hutsler and Casanova, 2016) was related to reduced lateral inhibition, bringing about changes in the spatial organization of neuronal activation and synaptic plasticity. In particular, center-surround (C/S) structures were proposed to change from a "Mexican hat" to a "stovepipe hat" profile (Casanova, 2006).

A key role in synaptic and microcircuit dysregulation has been suggested by NMDA receptor hyperfunction (Rinaldi et al., 2007). Important for the present case, NMDA receptor-mediated currents were increased in cerebellar granule cells of the IB2 (Islet Brain-2) KO mouse. These mice have a range of behavioral deficits reminiscent of Phelan McDermid syndrome and ASD (Phelan et al., 2001; Manning et al., 2004; Soorya et al., 2013; Kolevzon et al., 2014), in which autistic symptoms are associated with cerebellar functional deficits including a severe impaired motor performance and learning (Giza et al., 2010). IB2 (MAPK8IP2) is a scaffolding
protein enriched in the PSD, probably regulating signal transduction by protein kinase cascades, that operates inside the NMDA receptor interactome (Yasuda et al., 1999). Since NMDA receptor expression in granule cells is the highest among cerebellar neurons (Monaghan and Cotman, 1985) and has a profound impact on synaptic excitation and plasticity (D'Angelo et al., 1995; Armano et al., 2000; Sola et al., 2004; D'Errico et al., 2009), IB2 KO mice actually provide an ideal model to investigate cerebellar microcircuit alterations in ASD. In the cerebellar granular layer, granule cells receive excitatory synapses from mossy fibers and are inhibited by Golgi cells. The synaptic interaction between these neurons forms the granular layer microcircuit which, once activated by incoming spike bursts, generates responses organized in C/S (Mapelli and D'Angelo, 2007; Gandolfi et al., 2014). Here we show that the granular layer of IB2 KO mice is characterized by hyper-excitability and hyper-plasticity, which raise the E/I balance disrupting C/S structures and signal transfer at the input stage of cerebellum. The implications of these cerebellar microcircuit alterations for ASD pathogenesis are discussed.

**Methods**

All procedures were conducted in accordance with European guidelines for the care and use of laboratory animals (Council Directive 2010/63/EU) and approved by the Ethical Committee of Italian Ministry of Health (637/2017-PR).

**Genotyping and maintenance of IB2 KO mice**

Experiments were conducted on IB2^{+/+} (WT) and IB2^{-/-} (KO) mice obtained by crossing IB2^{+/+} parents, since IB2 KO are poor breeders, possibly reflecting the social deficit associated with IB2 deletion (Giza et al., 2010). The genotyping was conducted through PCR using four primers to detect wild-type and null alleles as previously described (Giza et al., 2010).
Slice preparation and solutions

The experiments reported in this paper have been conducted on 17- to 24-day-old (P0=day of birth) WT and IB2 KO mice of either sex. Mice were anesthetized with halothane (Sigma, St. Louis, MO) and killed by decapitation in order to remove the cerebellum for acute slice preparation according to a well-established technique (D'Angelo et al., 1995; Armano et al., 2000; Gall et al., 2005; Prestori et al., 2013; Nieuw et al., 2014). The vermis was isolated and fixed on the vibroslicer’s stage (Leica VT1200S) with cyano-acrylic glue. Acute 220 μm-thick slices were cut in the parasagittal plane in ice cold (2–3°C) Krebs solution containing (in mM): 120 NaCl, 2 KCl, 1.2 MgSO4, 26 NaHCO3, 1.2 KH2PO4, 2 CaCl2, and 11 glucose, equilibrated with 95% O2-5% CO2 (pH 7.4). Slices were allowed to recover at room temperature for at least 1h, before being transferred to a recording chamber mounted on the stage of an upright microscope (Zeiss, Oberkochen, Germany). The slices were perfused with oxygenated Kreb’s solution and maintained at 32°C with a Peltier feedback device (TC-324B, Warner Instrument Corp., Hamden, CT). Moreover, bath perfusion with Kreb’s solution was commenced before seal formation and was maintained until end of recording. Unless otherwise stated, Kreb’s solutions contained the GABA_A receptor antagonist SR95531 (gabazine, 10 μM; Abcam) (control condition). In some experiments, modified Kreb’s solutions were either Mg2+-free, gabazine free, or contained drugs to block excitatory glutamate receptors [2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX, 10 μM), D-(-)-2-Amino-5-phosphono-pentanoic acid (APV, 50 μM), 7-chlorokynurenic acid (50 μM)].

Experimental Design: Electrophysiological recordings

Whole-cell patch-clamp recordings were performed with Multiclamp 700B [-3dB; cutoff frequency (fc), 10 kHz], sampled with Digidata 1440A interface, and analyzed off-line with pClamp10 software (Molecular Devices, CA, USA). Patch pipettes were pulled from borosilicate
glass capillaries (Sutter Instruments, Novato, CA) and filled with different solutions depending on the specific experiments (see below). Mossy fiber stimulation was performed with a bipolar tungsten electrode (Clark Instruments, Pangbourne, UK) via a stimulus isolation unit. The stimulating electrode was placed over the central fiber bundle in the cerebellar lamina to stimulate the mossy fibers, and 200 μs step current pulses were applied at the frequency of 0.1-0.33 Hz (in specific experiments, paired-pulse stimulation at 20 ms inter-pulse was used). From a comparison with data reported in (Sharma and Vijayaraghavan, 2003; Giza et al., 2010; Sgritta et al., 2017), 1 or 2 mossy fibers were stimulated per granule cell in the experiments used for quantal analysis. Long-term potentiation (LTP) induction was obtained by a continuous stimulation of 100 pulses at 100Hz at −50 mV (HFS), as reported previously (Armano et al., 2000; Gall et al., 2005; D’Errico et al., 2009; Prestori et al., 2013). The stability of whole-cell recordings can be influenced by modification of series resistance (R_s). To ensure that R_s remained stable during recordings, passive electrode-cell parameters were monitored throughout the experiments. The granule cell behaves like a lumped electrotonic compartment and can therefore be treated as a simple resistive-capacitive system, from which relevant parameters can be extracted by analyzing passive current relaxation induced by step voltage changes. In each recording, once in the whole-cell configuration, the current transients elicited by 10 mV hyperpolarizing pulses from the holding potential of -70 mV in voltage-clamp mode showed a biexponential relaxation, with a major component related to a somatodendritic charging (Prestori et al., 2008). According to previous reports (D’Angelo et al., 1995; Silver et al., 1996; D’Angelo et al., 1999), the major component was analyzed to extract basic parameters useful to evaluate the recordings conditions and to compare different cell groups. Membrane capacitance (C_m) was measured from the capacitive charge (the area underlying current transients) and series resistance was calculated as R_s = τ_w/C_m. The membrane resistance (R_m) was computed from the steady-state current flowing after termination of the transient. The 3-dB cut-off frequency of the electrode-cell system was calculated as f_w = (2π • τ_w)\(^{-1}\). The data are reported in Table 1. In the cells considered for analysis, these values did not significantly change after 30 minutes attesting
recording stability. Cells showing variation of series resistance ($R_s$) >20% were discarded from analysis.

**Table 1**

*Granule cell excitability*

Patch pipettes had 7-9 MΩ resistance before seal formation with a filling solution containing (in mM): 126 potassium gluconate, 4 NaCl, 5 Heps, 15 glucose, 1 MgSO$_4$.7H$_2$O, 0.1 BAPTA-free, 0.05 BAPTA-Ca$^{2+}$, 3 Mg$^{2+}$-ATP, 0.1 Na$^+$-GTP, pH 7.2 adjusted with KOH. The calcium buffer is estimated to maintain free calcium concentration around 100 nM. Just after obtaining the cell-attached configuration, electrode capacitance was carefully cancelled to allow for electronic compensation of pipette charging during subsequent current-clamp recordings. At the beginning of each recording, a series of depolarizing steps was applied in voltage-clamp to measure the total voltage-dependent current of the granule cell (see Fig. 1C). Leakage and capacitance were subtracted using a hyperpolarizing pulses delivered before the test pulse (P/4 protocol). After switching to current-clamp, intrinsic excitability was investigated (see Fig. 1B) by setting resting membrane potential at -80 mV and injecting 800-ms current steps (from -4 to 22 pA in 2 pA increment). Membrane potential during current steps was estimated as the average value between 600 and 800 ms. Action potential frequency was measured by dividing the number of spikes by step duration.

*Post-synaptic currents*

Patch pipettes had 5-8 MΩ resistance before seal formation with a filling solution containing the following (in mM): 81 Cs$_2$SO$_4$, 4 NaCl, 2 MgSO$_4$, 1 QX-314 (lidocaine N-ethyl bromide), 0.1 BAPTA-free and 0.05 BAPTA-Ca$^{2+}$, 15 glucose, 3 Mg$^{2+}$-ATP, 0.1 Na$^+$-GTP, and 15
HEPES, pH adjusted to 7.2 with CsOH. The calcium buffer is estimated to maintain free calcium concentration around 100 nM. Synaptic currents elicited at 0.1 Hz were averaged and digitally filtered at 1.5 kHz off-line. IPSC and EPSC peak amplitude were taken at +10 and -70 mV to measure the GABA<sub>A</sub> and AMPA currents, respectively. In some experiments, NMDA current was directly measured at -70 mV in Mg<sup>2+</sup>-free solution in the presence of the AMPA receptor blocker, 10 µM NBQX (Sola et al., 2004). In LTP experiments, the acquisition program automatically alternated EPSC with background activity recordings (1 s and 9 s, respectively), from which mEPSCs were detected. After 10 min (control period), the recording was switched to current clamp (patch pipettes were filled with a K'-gluconate based solution) and high-frequency stimulation (HFS) was delivered to induce plasticity. Long-term synaptic efficacy changes were measured after 20 min. After delivering HFS, voltage-clamp at -70 mV was reestablished and stimulation was restarted at the test frequency. EPSCs and mEPSCs were digitally filtered at 1.5 kHz and analyzed off-line with pClamp10 software (Molecular Devices, Sunnyvale, CA). For both EPSC and mEPSC peak amplitude was computed. mEPSC detection was performed automatically with Mini Analysis Program (Synaptosoft, Inc. Decatur, GA) when their amplitude was 5-7 time the baseline noise S.D. (0.88 ± 0.03; n=8). These criteria and a further visual inspection of detected signals allowed us to reject noise artifacts.

In order to investigate the expression mechanism of long-term synaptic plasticity over a heterogeneous data set (Sola et al., 2004; Gall et al., 2005), a simplified version of quantal analysis was performed by measuring the mean (M) and standard deviation (S) of EPSC amplitude. EPSC changes, which do not strictly require that single synaptic connections are isolated, were obtained from M and S: the coefficient of variation, CV = S/M, the paired-pulse ratio, PPR = M<sub>2</sub>/M<sub>1</sub>, i.e. the ratio between the second and first EPSC amplitude in a doublet at 20 ms inter-pulse interval. The comparison between M and CV obtained before and after the induction of plasticity could be performed in the plot (CV<sub>2</sub>/CV<sub>A1</sub>)<sup>2</sup> vs. (M<sub>2</sub>/M<sub>1</sub>). Assuming binomial statistics, this plot has the property that the unitary slope diagonal separates points caused by changes in quantum content (m...
$= np$, with $n$ being the number of releasing sites and $p$ the release probability) from those caused by changes in quantum size ($q$). The inequality leads to a topological representation of neurotransmission changes (see Fig.7) and has been extensively used to interpret the plasticity mechanism (Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Sola et al., 2004; Rinaldi et al., 2008c; D’Errico et al., 2009; Sgritta et al., 2017). For an M increase:

(i) when $(\text{CV}_2/\text{CVA}_1)^2 > (M_2/M_1)$ both $n$ and $p$ can increase,

(ii) when $(\text{CV}_2/\text{CVA}_1)^2 = (M_2/M_1)$ only $n$ can increase,

(iii) when $(\text{CV}_2/\text{CVA}_1)^2 < (M_2/M_1)$ neither $n$ nor $p$ can increase implying an increase in $q$. A pure increase in $q$ will lie on the axis when $(\text{CV}_2/\text{CVA}_1)^2 = 1$.

Experimental Design: Voltage sensitive dye imaging (VSDi)

The stock solution for VSDi contained the dye Di-4-ANEPPS (Molecular Probes, Eugene, OR) dissolved in a Krebs-based solution containing 50% ethanol (Sigma) and 5% Cremophor EL (Sigma). Slices for optical recordings were incubated for 30 minutes in oxygenated Krebs solution added with 3% Di-4-ANEPPS stock solution and mixed with an equal volume of fetal bovine serum (Molecular Probes) to reach a final dye concentration of 2 mM (Vranesic et al., 1994). After incubation, the slices were rinsed with Krebs solution to wash out the dye that was not incorporated by the tissue, before being transferred to the recording chamber installed on an upright epifluorescence microscope (Slicescope, Scientifica Ltd, Uckfield, UK), equipped with a 20X objective (XLUMPlanFl 0.95 NA, water immersion; Olympus, Tokyo, Japan). The light generated by a halogen lamp (10V150W LM150, Moritex, Tokyo, Japan) was controlled by an electronic shutter (Newport corporation, Irvine, CA) and then passed through an excitation filter ($\lambda = 535 \pm 20$ nm), projected onto a dichroic mirror ($\lambda = 565$ nm) and reflected toward the objective lens to illuminate the specimen. Fluorescence generated by the tissue was transmitted through an absorption filter ($\lambda > 580$ nm) to the CCD camera (MICAM01, Scimedia, Brainvision, Tokyo, Japan). The whole imaging system was connected through an I/O interface (Brainvision) to a PC.
controlling illumination, stimulation and data acquisition. The final pixel size was 4.5x4.5μm with 20X objective. Full-frame image acquisition was performed at 0.5 kHz. Data were acquired and displayed by Brainvision software and signals were analyzed using custom-made routines written in MATLAB (Mathworks, Natick, MA). At the beginning of recordings, a calibration procedure was adopted to ensure homogeneity across experiments. The dynamic range of the CCD camera was calibrated by measuring background fluorescence and setting the average light intensity in the absence of stimulation to 50% of the saturation level. The background fluorescence was sampled for 50 ms before triggering electrical stimulation and was used to measure the initial fluorescence intensity ($F_0$). The relative fluorescence change ($\Delta F/F_0$) was then calculated for each time frame. The signal-to-noise ratio was improved by averaging 10 consecutive sweeps at the stimulus repetition frequency of 0.1 Hz.

VSDi data analysis

Fluorescence data collected by Brainvision acquisition software were filtered using both a cubic filter (3x3) and a spatial filter (3x3) embedded in the software, and then exported and processed in Matlab. The resulting files were a series of matrices each representing a temporal frame of the acquired trace. Using appropriate Matlab routines written ad hoc, single matrices representing the peak value of granular layer responses to electrical stimulation were obtained. These maps containing the information on the signal peak amplitudes and their spatial origin were used for comparison of control condition and different treatments, as detailed below. Data were reported as mean ± SEM. Statistical significance was assessed using unpaired Student's t test unless otherwise stated. For the analysis of the amount and spatial distribution of the NMDA receptor component of excitation in the cerebellar granular layer of WT and IB2 KO mice, responses to electrical stimulation of the mossy fibers were recorded in control and after perfusion of the NMDA receptor blocker APV (50 μM). The average map of APV effect on signal amplitudes was subtracted to the control map, to unveil the contribution of the NMDA receptors. The spatial
distribution of the NMDA receptor-mediated depolarization was revealed by averaging each experimental map on the peak of NMDA receptor component in each case. Whenever spatial maps obtained from different experiments were averaged, the corresponding slices were aligned along the mossy fiber bundle. For the analysis of the excitatory/inhibitory (E/I) balance and spatial distribution of excitation and inhibition in the granular layer, similar experiments were carried out, recording the responses to MFs stimulation before and after the perfusion of the GABA\textsubscript{A} receptor antagonist SR95531 (gabazine; 10 \textmu{M}). This approach allows to reconstruct a map of regions with prevailing excitation (E) compared to regions showing prevailing inhibition (I) (Mapelli and D'Angelo, 2007; Gandolfi et al., 2014). In this case, the E map was constructed on the control responses (where the response is available only in the regions where excitation prevails over inhibition), while the I map was constructed subtracting the maps after SR95531 perfusion to the control maps (unveiling the regions where, before SR95531 perfusion, excitation was prevented by inhibition). Both E and I maps were normalized to 1, and the E/I balance maps were obtained as (E-I)/E. The C/S organization of excitation and inhibition was evident averaging the E/I maps in each experiment on the peak of excitation in controls. For the analysis of the amount and spatial distribution of LTP and LTD in the granular layer, plasticity maps were obtained by comparing responses amplitudes before and after the plasticity induction through a HFS delivered to the mossy fiber bundle. The C/S spatial organization of LTP and LTD was unveiled by averaging each plasticity maps from different experiments on the peak of maximum LTP.

Statistical analysis

Results are reported as mean ± SEM and compared for their statistical significance by unpaired Student’s test (unless otherwise stated; a difference was considered significant at p < 0.05).
Results

In the cerebellum granular layer, there are three main mechanisms controlling the E/I balance of granule cells (Nieus et al., 2014): granule cells intrinsic excitability, mossy fiber glutamatergic excitation, Golgi cell GABAergic inhibition (Mapelli et al., 2014) (Fig. 1A). Here, these properties have been compared in turn between IB2 KO and WT mice. In patch-clamp whole-cell recordings in acute cerebellar slices, there were no significant differences in resting membrane potential between IB2 KO and WT cerebellar granule cells but there was a small but significant difference in passive membrane properties (see below and Table 1).

Enhanced intrinsic excitability in IB2 KO granule cells

In whole-cell current-clamp recordings, both WT and IB2 KO granule cells were silent at rest and responded to current steps with fast repetitive spike discharges that increased their frequency almost linearly with stimulus intensity (D'Angelo et al., 1995; Brickley et al., 1996; D'Angelo et al., 1998; Rossi et al., 1998; Armano et al., 2000; Cathala et al., 2003; Prestori et al., 2008) (Fig. 1B). However, IB2 KO granule cells showed higher discharge frequency compared to WT granule cells both at low current injection [12 pA: WT = 4.1 ± 0.1 Hz (n=6); IB2 KO = 48.1 ± 14.2 Hz (n=8); p=0.017, unpaired t-test] and at high current injection [20 pA: WT = 39.2 ± 9.5 Hz (n=6); IB2 KO = 93.7 ± 16.0 Hz (n=8); p=0.014, unpaired t-test], shifting the frequency-intensity plot toward the left (Fig. 1B). In the same experiments, whole-cell currents elicited by depolarizing voltage steps and differed in WT and IB2 KO granule cells (Fig. 1C). The transient inward current density (corresponding to a fast Na⁺ current) (Magistretti et al., 2006) was significantly larger in IB2 KO compared to WT granule cells. The transient and persistent outward currents density (comprising A-type, delayed rectifier, and calcium-dependent K⁺ currents) (Bardoni and Belluzzi, 1994) were also significantly larger in IB2 KO compared to WT granule cells. It should be noted
that input membrane capacitance ($C_m$) was slightly but significantly smaller and that input membrane resistance $R_m$ was slightly but significantly higher in IB2 KO compared to WT granule cells (Table 1). Therefore, differences in both current density changes and passive membrane properties support the concept that IB2 KO granule cells were more excitable than WT granule cells.

In order to investigate if tonic currents or background synaptic activity (Mitchell and Silver, 2000; Rossi et al., 2002; Mitchell and Silver, 2003) could influence the frequency-intensity plots, experiments have also been performed in the presence of excitatory receptor blockers (Fig. 1B). In WT granule cells, the perfusion of AMPA and NMDA receptor antagonists did not cause any significant changes in the frequency-intensity plot, which showed a similar discharge frequency compared to control both at low current injection [12 pA: 5.3 ± 3.0 Hz (n=8) vs, 4.1 ± 0.1 Hz (n=6); $p = 0.76$, unpaired $t$-test] and at high current injection [20 pA: 26.6 ± 5.6 Hz (n=8); versus 39.2 ± 9.5 Hz (n=6); $p = 0.28$, unpaired $t$-test; Fig. 1B]. Interestingly, in IB2 KO granule cells, the perfusion of AMPA and NMDA receptor antagonists caused a significant reduction in excitability, so that the frequency-intensity plot did no longer differ from that of WT granule cells. The discharge frequency was similar both at low current injection [12 pA: WT = 5.3 ± 3.0 Hz (n=8); IB2 KO = 2.0 ± 1.5 Hz (n=6); $p=0.34$, unpaired $t$-test] and at high current injection [20 pA: WT = 26.6 ± 5.6 Hz (n=8); IB2 KO = 35.7 ± 12.0 Hz (n=6); $p=0.52$, unpaired $t$-test; Fig. 1B]. Moreover, in IB2 KO granule cells, in the presence of glutamate receptor antagonists, the outward current densities were not different from WT granule cells (Fig. 1C). These data suggest that, in IB2 KO mice, the increased granule cell responsiveness could be related to excitatory receptors activation by tonic glutamate and consequent deregulation of membrane ionic currents (e.g. see (Sah et al., 1989; Nieus et al., 2006; Le Meur et al., 2007).
Similar AMPA and GABA<sub>A</sub> but increased NMDA receptor mediated currents at IB2 KO granule cell synapses

Mossy fiber stimulation is known to elicit EPSCs directly through mossy fiber activation and IPSCs indirectly through activation of Golgi cells (cfr. Fig.1A) (Cathala et al., 2003; Cesana et al., 2013; Nieus et al., 2014). Postsynaptic currents were recorded from granule cells both at -70 mV and +10 mV in order to isolate the excitatory (EPSC) from inhibitory (IPSC) component. This technique was reported previously (Mapelli et al., 2009; Nieus et al., 2014). It should be noted that, at -70 mV, NMDA receptor-mediated currents are blocked by Mg<sup>2+</sup>, so that the EPSC is almost purely AMPA receptor-mediated. In the present experiments, the AMPA-EPSC peak (WT = -38.1 ± 7.1 pA, n=13 vs. IB2 KO = -34.1 ± 5.7, n=7; p=0.66) and the GABA<sub>A</sub>-IPSC peak (WT = 45.4 ± 8.4 pA, n=13 vs. IB2 KO = 51.7 ± 13.4, n=7; p=0.69) showed similar amplitude in WT and IB2 KO mice (Fig. 2A). Accordingly, no differences were observed in the AMPA-EPSC/GABA<sub>A</sub>-IPSC ratio in granule cells (WT = 0.95 ± 0.15, n=13 vs. IB2 KO = 0.86 ± 0.19, n=7; p=0.71; Fig. 2B).

In a different series of recordings, the NMDA EPSC was elicited in isolation at −70 mV in Mg<sup>2+</sup>-free solution in the presence of AMPA and GABA<sub>A</sub> receptor blockers (10 μM NBQX and 10 μM SR95531, respectively; Fig. 2A). The NMDA-EPSC peak was enhanced in IB2 KO synapses (WT = -37.0 ± 5.1 pA, n=6 vs. IB2 KO = -95.3 ± 17.7, n=5; p=0.03) by 2.5 times. These results confirm the alteration in NMDA EPSC amplitude reported previously (Giza et al., 2010).

In aggregate, the similarity of the AMPA-EPSC and GABA<sub>A</sub>-IPSC, along with the large increase of the NMDA-EPSC, suggest that the excitatory/inhibitory (E/I) balance in IB2 KO mice will move in favor of excitation in conditions in which the NMDA channels are physiologically unblocked by depolarization.

********** Figure 2 **********

Increased excitation in C/S structures of IB2 KO granular layer are driven by NMDA currents
In order to obtain a physiological assessment of the E/I balance and of the NMDA current contribution, we used voltage-sensitive dye imaging (VSDi). VSDi allows to generate maps of electrical activity and to investigate the spatial distribution of granular layer responses following mossy fiber stimulation (Mapelli et al., 2010). In particular, VSDi, coupled with selective pharmacological blockade of synaptic receptors, can reveal the relative role of synaptic inhibition and of NMDA receptors (Gandolfi et al., 2015).

A first set of VSDi recordings was performed by subtracting control activity maps from those obtained after GABA_A receptor blockade with 10 μM SR95531 (Fig. 3; see Methods for details). In agreement with previous observations, the granular layer response to mossy fiber stimuli self-organized in center/surround (C/S) structures characterized by a "Mexican hat" profile, with an excitation core surrounded by inhibition (Mapelli and D'Angelo, 2007; Solinas et al., 2010; Gandolfi et al., 2014; Gandolfi et al., 2015) (Figs 3A,B). The C/S distribution was maintained in the IB2 KO granular layer but with striking differences. (i) Excitation was enhanced generating larger cores compared to WT (core diameter: WT = 12.9 ± 1.7 μm vs. IB2 KO = 29.5 ± 4.9 μm, n=5 for both; p=0.0106) (Fig. 3C). (ii) Inhibition was weaker in the surround (WT/KO ratio lWT/KO = 2.83 ± 0.17, n=5). (iii) Granular layer areas showing excitation were consequently larger in IB2 KO than WT mice (WT = 49.9 ± 3.1% vs. IB2 KO = 58.8 ± 2.1%, n=5 for both; p=0.0468; Fig. 3C). As a result, the altered C/S organization in IB2 KO showed larger excitation cores with poor inhibitory surrounds, shifting from "Mexican hat" to the so-called "stovepipe hat" shape (see Fig. 3B).

A second set of VSDi recordings was performed by subtracting control activity maps from those obtained after NMDA receptor blockade with 50 μM APV (Fig. 4; see Methods for details). As expected from the increased NMDA receptor-mediated current reported in Fig. 2, the NMDA receptor-mediated component of the VSDi signal was larger in IB2 KO than WT granular layers (ratio KO/WT = 2.16 ± 0.29, n=5 for both). The maps showing the spatial organization of the NMDA receptor contribution to the excitatory response were similar to the C/S organization shown in Fig. 3, with peaks of NMDA receptor contribution in cores with a diameter of 26.1 ± 1.7 μm in...
IB2 KO vs. 18.9 ± 1.6 μm in WT; n=5 for both; p=0.015 (Figs. 4A,B). Interestingly, since during VSDi membrane potential remains unclamped allowing voltage-dependent NMDA channel unblock during depolarization, these maps provide information about the non-linear contribution of NMDA currents. This result supported the hypothesis that the enhanced NMDA receptor-mediated transmission revealed in Fig. 2 was indeed a key player in determining the C/S and E/I alteration in IB2 KO granular layer.

********** Figure 3, 4 *********

Enhanced long-term potentiation at the IB2 KO mossy fiber-granule cell relay

Mossy fiber-granule cell LTP is NMDA receptor-dependent through the synaptic control of postsynaptic intracellular calcium elevation (D'Angelo et al., 1999; Maffei et al., 2003; Gall et al., 2005; D'Errico et al., 2009). The impact of elevated NMDA receptor-dependent neurotransmission on LTP induction in IB2 KO mice was evaluated using a continuous high-frequency stimulation train (HFS; Fig. 5A) delivered from the holding potential of -50 mV in current-clamp (Gall et al., 2005; D'Errico et al., 2009). During HFS, IB2 KO generated more spikes than WT granule cells (WT = 23.5 ± 5.3, n=12 vs. IB2 KO = 54.2 ± 11.4, n=9; p=0.015; Figs. 5A,B), in line with the enhancement in NMDA currents (D'Angelo et al., 2005) and in intrinsic firing reported above (cf. Figs 1 and 2). After HFS, the changes were evaluated over at least 25 min after HFS.

The AMPA EPSC increased both in WT and IB2 KO mice and remained potentiated throughout the recordings (Fig. 5C). The increase in amplitude of AMPA-EPSCs was ~5-fold larger in IB2 KO than WT mice (WT = 20.4 ± 4.2 %, n=12 vs. IB2 KO = 102.4 ± 34.9 %, n=9; p=0.047; Fig. 5D).

Intrinsic excitability increased more in WT than in IB2 KO mice (Fig. 6A,B). The current needed to generate spikes (current threshold) decreased significantly compared to control in WT granule cells (-42.8 ± 7.7%, n=6; p=0.0055) but not in IB2 KO granule cells (-8.6 ± 14.4%, n=8; p=0.07; Fig. 6B). Moreover, the increase in spike frequency was less pronounced in IB2 KO than
WT granule cells (WT = 102.6 ± 19.3%, n=6 vs. IB2 KO = 21.1 ± 8.7%, n=8; p=0.032; Fig. 6B). A possible explanation of this effect could be that granule cell intrinsic excitability was already increased in IB2 KO granule cells (cf. Fig.1B), such that the level of IB2 KO granule cell excitability in control was similar to that in WT granule cells after potentiation (Fig. 6B).

As a further control, we monitored the apparent granule cell input resistance (Fig. 6C,D) by measuring the response to small current steps (causing about 10 mV potential changes) either below -70 mV (R_{in-low}) or above -70 mV (R_{in-high}) (Armano et al., 2000). After HFS, R_{in-high} rapidly increased in both WT and IB2 KO mice, following a similar time course and remained potentiated throughout the recordings (at least 20 min after HFS; average time courses are shown in Fig. 6D). At 20 min after HFS, R_{in-high} increase was 67.8 ± 16.5% (n=8) (p=0.0014) in WT and 46.9 ± 9.0% (n=10) in IB2 KO mice (p=0.00012). This change was likely to contribute to the increased intrinsic excitability in both WT and IB2 KO. It should be noted that R_{in-low} remained unchanged in both WT and IB2 KO, providing an internal control for recording stability (Fig. 6C,D).

********** Figures 5, 6 **********

LTP dependence on NMDA and GABA-A receptors in IB2 KO granule cells

The role of NMDA receptors in the induction of long-term potentiation between mossy fiber-granule cell synapse is well established (D'Angelo et al., 1999; Armano et al., 2000; Maffei et al., 2002; Sola et al., 2004; D'Angelo et al., 2005; D'Errico et al., 2009). In order to test whether LTP in these experiments was NMDA receptor dependent, we first evaluated whether HFS could induce LTP when NMDA receptors were blocked. When the NMDA receptors antagonists, D-APV (50 μM) and 7-Cl-Kyn acid (50 μM), were added to the extracellular solution, HFS failed to induce LTP in both WT and IB2 KO mice (WT = 0.23 ± 5.9%, n=7; p = 0.89 vs. IB2 KO = -22.9± 4.9%, n=6; p=0.03; Fig. 7A). Interestingly, a significant LTD appeared in IB2 KO mice.
NMDA-receptor-dependent LTP in the granular layer strongly depends on depolarization during induction and activation of GABA_\text{A} receptors, by limiting depolarization, can indeed prevent LTP (Armano et al., 2000; Maffei et al., 2002). In order to test whether LTP in these experiments could be prevented by GABA_\text{A} receptors, in a set of experiments the GABA_\text{A} receptors antagonist gabazine was omitted from the extracellular solution. Figure 7B shows the time course of EPSC changes. The removal of gabazine prevented LTP in both WT mice and IB2 KO mice (WT = -12.6 ± 8.6%, n=7; p =0.38 vs. IB2 KO = -36.4 ± 8.3%, n=7; p=0.005). Interestingly, a significant LTD appeared in IB2 KO mice.

These results indicate that, in IB2 KO mice like in WT mice, LTP induction requires NMDA receptor activation and can be prevented by GABA_\text{A} receptor activation. However, in IB2 KO mice, NMDA and GABA_\text{A} receptor blockers unveiled an underlying LTD.

********** Figure 7 **********

Different mechanisms of LTP expression

LTP expression was first assessed by analyzing changes in EPSC amplitude, variability (CV), and paired-pulse ratio (PPR) (Fig. 8A). The paired-pulse ratio (PPR) of EPSCs is generally considered to reflect changes in the probability of transmitter release in a pair of stimuli (Zucker and Regehr, 2002), while the coefficient of variation (CV) of EPSCs is a readout of presynaptic variability of quantal transmitter release upon repeated stimulation normalized by the mean (Malinow and Tsien, 1990; Manabe et al., 1993). In the recordings used for PPR and CV analysis, after HFS, the EPSCs showed a significant increase in WT (18.2 ± 3.4; n=8; p= 0.012) and IB2 KO mice (106.8 ± 51.8%; n=5; p= 0.05), while PPR (interstimulus interval 20 ms) showed a significant reduction in WT (-19.6 ± 9.3 %, n=8; p = 0.033) but not in IB2 KO (-6.7 ± 3.3 %, n=5; p = 0.1). Interestingly, CV significantly decreased in both WT and IB2 KO (WT = -28.3 ± 6.7, n=12; p=0.002; IB2 KO = -30.0 ± 8.0, n=9; p = 0.012).
release was increased not just in WT (Sola et al., 2004) but also in IB2 KO mice, although with
some difference (see below).

The CV and PPR analysis cannot stand alone in determining the changes that could affect
the neurotransmission process (Yang and Calakos, 2013). A further way to assess whether EPSC
changes depend on the number of releasing sites \((n)\), release probability \((p)\) or quantum size \((q)\) is to
plot \((CV_2/CV_1)^2\) versus \((M_2/M_1)\) (Bekkers and Stevens, 1990; Malinow and Tsien, 1990) (Fig. 8B).
The WT experimental data points were distributed homogenously in the quadrant corresponding to
\(p/n\) increase, with no point falling in the regions of a pure \(n\) or \(q\) change. Conversely, the IB2 KO
experimental dataset was heterogeneously distributed over regions of \(p\), \(n\) or \(q\) increase. These data
distributions suggested that multiple presynaptic and postsynaptic mechanisms contributed to
determine LTP at IB2 KO mossy fiber-granule cell synapses.

A second experimental approach to quantal analysis is to examine miniature postsynaptic
currents (mEPSCs) before and after LTP induction (Fig. 8C,D) (Kullmann and Nicoll, 1992; Wyllie
et al., 1994; Malgaroli et al., 1995). This method is especially useful at multi-quantal release
synapses like here (Sola et al., 2004; Saviane and Silver, 2006) and can allow to distinguish
between an increase in quantum content \((p\) or \(n)\) or quantum size \((q)\). Since here mEPSCs accounted
for the whole spontaneous mossy fiber activity, in LTP experiments mEPSCs were recorded
without TTX and were used to characterize the LTP expression mechanism (Sola et al., 2004).
Moreover, in order to prevent mEPSC changes from being obscured by the contribution of non-
potentiated synapses, we activated as many synapses as possible by raising stimulus intensity.
Indeed, in these recordings, the EPSCs \([-59.0 \pm 11.0\ pA\ (n=4)\) in WT and -55.0 \pm 14.9\ pA\ (n=4)\) in
IB2 KO mice] were about twice as large than those measured in Fig. 2 [(by comparison with single
fiber EPSCs measured in similar recording conditions, this corresponded to activation of two to
three mossy fibers (Sola et al., 2004)]. After HFS, the EPSCs increased (WT = 19.0 \pm 2.0\%, n=4; \(p
= 0.02\) vs. IB2 KO = 93.6 \pm 49.7, n=4; \(p = 0.02\) confirming larger LTP induction in IB2 KO than
WT mice (cf. Fig. 8A). In the same recordings, mEPSCs amplitude did not vary in WT granule cells
(3.3± 3.7 %, n=4; p = 0.4) but showed significant increase in IB2 KO granule cells (28.9 ± 5.66 %, n=4; p = 0.016). Conversely, mEPSC frequency showed a significant increase in WT granule cells (46.1± 12.9 %, n=4; p = 0.016) but did not show any significant changes in IB2 KO granule cells (-16.9± 6.0 %, n=4; p = 0.11). Therefore, mEPSC analysis indicated that, while WT granule cells showed an increase in quantum content [(as reported previously in rats (Sola et al., 2004)], IB2 KO granule cells showed an increased quantum size.

In aggregate, these results confirm that LTP in wild type mice depends almost exclusively on increased neurotransmitter release probability (>p) and suggest that LTP in IB2 KO mice rests on a more complex mechanism including both changes in quantum content (>p, n) and quantum size (>q).

********** Figure 8 **********

Altered spatial distribution of LTP and LTD in the granular layer of IB2 KO mice

Given the enhanced LTP magnitude (cf. Fig. 5) and the altered C/S organization in IB2 KO granular layer (cf. Fig. 3), VSDi experiments were conducted in order to unravel possible alterations in the spatial distribution of LTP and LTD in IB2 KO granular layer. As recently shown using the same technique, the spatial distribution of areas undergoing LTP and LTD in the cerebellar granular layer displays a C/S-like organization, with LTP in the core and LTD in the surround (Gandolfi et al., 2015). The investigation of this feature in WT granular layer revealed a similar organization. Interestingly, the C/S organization of core-LTP and surround-LTD in IB2 KO granular layers showed a shape alteration with larger LTP cores and thinner LTD surrounds (Fig.9).

The analysis of the granular layer areas with LTP and LTD revealed several abnormalities with respect to WT: i) LTP magnitude in the center was higher (WT = 28.4 ± 3.3% vs. IB2 KO =109.4±6.7%, n=6 for both, p=8x10^-6); ii) LTP total area underwent an impressive increase (WT = 3.3 ± 1.5% vs. IB2 KO = 10.2 ± 3.3%, n=6 for both; p=0.047), iii) LTD total area and magnitude were decreased (WT = 91.4±1.9% vs. IB2 KO = 81.3±3.7%, n=6 for both; p=0.037; total LTD
magnitude: \( WT = -34.9 \pm 2.8\% \) vs. IB2 KO = \(-24.9 \pm 2.6\% \), \( n=6 \) for both, \( p=0.0026 \), and iv) the C/S
shape showed a significant change in favor of LTP. In particular, the LTP-center was broader in IB2
KO compared to WT (core diameter: \( WT = 8.4 \pm 0.7 \ \mu m \) vs. IB2 KO = \( 32.0 \pm 4.1 \ \mu m \), \( n=6 \)
respectively; \( p=0.0005 \)), and the LTD in the surround was less deep (\( WT = -37.3\pm1.5\% \) vs. IB2 KO
\( = -22.0\pm2.5\%; \ n=6 \) for both; \( p=0.0004 \)) (Fig. 9 C,D).

********** Figure 9 **********

Discussion

The main observation of this paper is that profound alterations in signal processing occur at
the input stage of cerebellum in an ASD model, the IB2 KO mouse. Intrinsic excitability, synaptic
transmission and synaptic plasticity in granule cells were enhanced in the absence of apparent
compensation by the inhibitory circuit, causing a net increase in E/I balance. This in turn changed
the spatial organization of neuronal responses, such that the core in C/S structures predominated
over the inhibitory surround and LTP spread over larger areas.

Granule cell hyper-functioning and the NMDA receptor-dependent current

In IB2 KO mice, cerebellar granule cells were hyper-functioning. Enhanced synaptic
transmission appeared as a 2.4 times larger spike emission in response to high-frequency input
bursts and was clearly correlated with larger NMDA receptor-mediated currents and increased
intrinsic excitability. Enhanced intrinsic excitability appeared as a 2.1-7.1 (depending on current
injection) higher efficiency in generating spikes during current injection and was correlated with
larger Na\(^+\) and K\(^+\) membrane currents. Enhanced synaptic plasticity was manifest as a 5.3 times
larger LTP compared to that normally measured at the mossy fiber - granule cell synapse (Prestori
et al., 2008; Prestori et al., 2013). While normal LTP is almost entirely sustained by increased
neurotransmitter release probability (Sola et al., 2004; D’Errico et al., 2009), IB2 KO LTP was expressed through a compound pre- and postsynaptic mechanism. This was consistently indicated by the increase in minis amplitude (>$q$) and decrease in EPSC PPR (> $n$, $p$) and confirmed by the ubiquitous distribution of points in the $(CV_2/CV_1)^{-2}$ vs. $(M_2/M_1)$ plot. The intervention of a postsynaptic expression mechanism was key to explain the neurotransmission increase in IB2 KO mice granule cells (~120%), which exceeds the theoretical limit of presynaptic expression alone (~60%; from (Sola et al., 2004)).

Interestingly, the whole set of alterations was likely to reflect, directly or indirectly, the NMDA receptor-mediated current enhancement occurring at the mossy fiber – granule cell synapse. In IB2 KO mice, the NMDA synaptic current of granule cells was increased by about 2.5 times, as anticipated by (Giza et al., 2010), while the AMPA receptor-mediated current was unaltered. During bursts, the granule cell NMDA current is known to exert a strong depolarizing action entraining a regenerative cycle (D’Angelo et al., 2005), in which depolarization removes NMDA channel unblock further increasing the NMDA current. The combination of this effect with enhanced intrinsic excitability could easily explain the enhanced synaptic transmission characterizing IB2 KO granule cells. In turn, enhanced NMDA receptor activation could also promote stronger plasticity of synaptic transmission and intrinsic excitability (Armano et al., 2000; Gall et al., 2005). An enhanced tonic NMDA receptor activation (Rossi et al., 2002) could also increase intrinsic excitability, possibly through facilitated NMDA receptor activation by ambient extracellular glutamate (Sah et al., 1989; Nieus et al., 2006; Le Meur et al., 2007) regulating intracellular cascades and ionic voltage-dependent mechanisms (Cohen et al., 1996; Jugloff et al., 2000; Marks and Fadool, 2007; Mulholland et al., 2008).

**Functional alterations of the granular layer microcircuit**

Given the absence of changes in synaptic inhibition, the enhancements in excitatory synaptic transmission and intrinsic excitability provide an explanation for the remarkable increase in E/I
balance, for the prevalence of core over surround in C/S responses and for the extension of the LTP territory. The C/S organization of the cerebellum granular layer depends on the balance between granule cell excitation and Golgi cell inhibition (Mapelli and D'Angelo, 2007). Here, the strong enhancement of the NMDA current could effectively counteract inhibition (Nieus et al., 2014) extending the core and changing the C/S from "Mexican hat" to "stovepipe hat" shape. The elevated input resistance and intrinsic excitability of IB2 KO granule cells could collaborate with elevated NMDA receptor-dependent transmission to spatially expand the excitatory footprint and zone of LTP. The consequences of NMDA receptor hyperfunctioning on the E/I balance and C/S changes could be further analyzed using realistic mathematical models of the granular layer (Solinas et al., 2010; Sudhakar et al., 2017).

NMDA receptor expression in granule cells is the strongest of cerebellum (Monaghan and Cotman, 1985) and is reasonable to speculate that a damage there could have a high impact on ASD pathogenesis. Although granular layer circuit alterations were uncompensated leading to a net E/I increase, some changes downstream might have a compensatory meaning. For example, in IB2 KO mice, the thinner molecular layer, the simplified dendritic tree and the smaller climbing fiber responses of Purkinje cells (Giza et al., 2010), may limit the impact of granular layer overexcitation. Another effect that might be compensatory, is the emergence of an NMDA receptor-independent form of mossy fiber – granule cell LTD, that might help counterbalancing the excess LTP at the same synapse when GABA_A receptor-dependent inhibition is engaged (see Fig. 7).

Comparison of alterations with other circuits and ASD models

The alterations observed in the cerebellum granular layer of IB2 KO mice resemble in some respects those observed in other brain structures of ASD mice. An enhanced NMDA receptor-mediated neurotransmission was proposed to cause hyper-reactivity and hyper-plasticity in the somatosensory cortex (Rinaldi et al., 2007; Rinaldi et al., 2008a), in pyramidal neurons of the medium prefrontal cortex (Rinaldi et al., 2008b) and in the amygdala (Markram et al., 2008).
Interestingly, hyper-reactivity and hyper-plasticity were correlated with enhanced E/I balance in relation with enhanced NMDA receptor-mediated neurotransmission (Markram et al., 2008). Therefore, our results support the concept that enhanced NMDA receptor-mediated neurotransmission is a common bottleneck for ASD pathogenesis in different brain areas, including the cerebellum. The change of C/S shape from "Mexican hat" to "stovepipe hat" is especially interesting in view of the ASD hypothesis developed for cortical minicolumns, the fundamental module of the neocortex (Casanova et al., 2002; Casanova et al., 2006; Hutsler and Casanova, 2016). The histological analysis postmortem of minicolumns in ASD patients has revealed reduced size and altered neuronal organization suggesting that lateral inhibition was reduced. In the C/S of the cerebellum granular layer, the effectiveness of lateral inhibition was indeed reduced by the increased intensity and extension of the excitation core. Therefore, a reduced effectiveness of surround inhibition of cortical and cerebellar modules may be a common trait of the disease in different brain microcircuits. The picture may be complicated by the interaction between causative, compensatory and developmental factors. For example, in Gabrb3 mutants, an increased metabotropic glutamate receptor activation in deep cerebellar nuclei has been proposed to prevent the downstream propagation of effects and to protect from ASD in males (Mercer et al., 2016).

**Possible consequences of alterations on cerebellar functioning**

The cerebellar granular layer has been proposed to perform expansion recoding and spatial pattern separation of input signals (Marr, 1969), which can be regulated by long-term synaptic plasticity at the mossy fiber - granule cell relay (Hansel et al., 2001; D'Angelo and De Zeeuw, 2009; D'Angelo, 2014). In IB2 KO mice, mossy fiber burst retransmission was enhanced and the effect could be further amplified by LTP (Nieuw et al., 2006). Moreover, the excited areas were broader and poorly limited by surround inhibition. Therefore, in the granular layer microcircuit expansion recoding and spatial pattern separation could be compromised. Moreover, IB2 KO mice showed a delayed transmission at climbing fiber-Purkinje cell synapse (Giza et al., 2010). Altogether, these
alterations could alter coincidence detection and deregulate LTD at parallel fiber - Purkinje cells synapses (Koekkoek et al., 2005; Baudouin et al., 2012; Piochon et al., 2014; Peter et al., 2016; Piochon et al., 2016). These functional modifications might impair the processes of novelty detection and attention switching (D'Angelo and Casali, 2013) toward new environmental or internal cues normally performed by associative areas (especially prefrontal), contributing to generate the combination of cerebellar and ASD symptoms presented by IB2 KO mice.

Conclusions

The complex derangement of signal processing and plasticity in the cerebellum granular layer of IB2 KO mice supports a causative role of cerebellum in ASD pathogenesis. NMDA receptor dependence and microcircuit alterations resembled the hallmarks reported for cortical minicolumns, including synaptic hyper-reactivity, synaptic hyper-plasticity, increased E/I balance and C/S changes. In the cerebellum, these alterations may play a crucial role in generating ASD as well as motor symptoms. The cerebellum forms multiple closed-loop circuits with cerebral cortical regions that underlie movement, attention, language, social processing and executive control (Snider and Eldred, 1951; Middleton and Strick, 2000; Habas et al., 2009; Strick et al., 2009; Kellermann et al., 2012; D'Angelo and Casali, 2013; Sokolov et al., 2014; Palesi et al., 2017) providing the anatomical substrate by which cerebellar dysfunction could contribute to ASD (Schmahmann, 2004; Schmahmann et al., 2007; Schmahmann, 2010; D'Angelo and Casali, 2013; Rogers et al., 2013; D'Mello and Stoodley, 2015; Stoodley et al., 2017). Future neuroimaging studies may be used to localize cerebellar activation in ASD (Stoodley et al., 2017) determining how cerebellar abnormalities combine with those occurring in other brain regions (Bolduc and Limperopoulos, 2009; Limperopoulos et al., 2009; Bolduc et al., 2011; Wang et al., 2014; Hampson and Blatt, 2015; Mosconi et al., 2015).

Legends

26
Table 1. The data were obtained using K-gluconate intracellular solution and analyzing current transient elicited by 10 mV voltage-clamp steps delivered from the holding potential of -70 mV. The number of observations indicated and statistical significance is reported in comparison with IB2 KO granule cells. *p<0.05; ***p<0.001, unpaired t test.

Figure 1. Granule cell excitable properties.

(A) Schematic representation of cerebellar circuit. Mossy fibers (mf) contact granule cells (GrC) and Golgi cell (GoC) dendrites. Axons of GrCs, the parallel fibers (pf), activate Golgi cells which inhibit GrCs through feedforward and feedback inhibitory loops.

(B) Granule cell electroresponsiveness in presence of 10 μM gabazine (control condition) and glutamate receptor antagonists (10 μM NBQX, 50 μM D-APV and 50 μM 7-Cl-Kyn acid). Voltage responses were elicited from -80 mV using step current injection. The plots show the relationships between average spike frequency over 800 msec and the injected current intensity both for WT and IB2 KO granule cells. Data are reported as mean ± SEM.

Linear fits in control condition (dashed lines): WT x-intercept 11.7 ± 0.8 pA, slope 5.7 ± 0.9 spike/pA (n=6); IB2 KO x-intercept 5.5 ± 2.5 pA (p = 0.04), slope 6.5 ± 0.5 spike/pA (n=8; p = 0.46). Data are reported as mean ± SEM.

Linear fits in presence of glutamate receptor antagonists (dashed lines): WT x-intercept 11.0 ± 1.6 pA, slope 3.6 ± 0.4 spike/pA (n=8); IB2 KO x-intercept 13.1 ± 1.8 pA (p = 0.4), slope 5.6 ± 1.15 spike/pA (n=6; p = 0.15).

(C) Voltage-activated inward and outward currents in granule cells. The histograms compare inward and outward current densities (normalized by input membrane capacitance, C_m) measured at -40 mV and +20 mV in WT and IB2 KO mice in presence of 10 μM gabazine (control condition) and glutamate receptor antagonists (10 μM NBQX, 50 μM D-APV and 50 μM 7-Cl-Kyn acid). Data are reported as mean ± SEM; *p<0.05.
Figure 2. Evoked excitatory and inhibitory currents in granule cells.

(A) Synaptic currents in WT and IB2 KO granule cells. The EPSC\textsubscript{AMPA} and IPSC are recorded from the same cells at the holding potential of -70 mV (averaging of 30 consecutive traces) and at +10 mV (averaging 10 consecutive traces), respectively. The EPSC\textsubscript{NMDA} are recorded in different cells at -70 mV in Mg\textsuperscript{2+}-free extracellular solution in presence of the AMPA receptor antagonist, 10 μM NBQX (averaging of 30 consecutive traces).

(B) IPSC/EPSC ratios at mossy fiber–granule cell synapses in WT and IB2 KO mice. The plot shows the amplitude of EPSC\textsubscript{AMPA} and IPSC in the same cells for WT and IB2 KO mice (open symbols are mean ± SEM). The histogram compares the average EPSC\textsubscript{AMPA}/IPSC ratio and EPSC\textsubscript{NMDA}/IPSC ratio in WT and IB2 KO mice Data are reported as mean ± SEM;; **p<0.01.

Figure 3. Excitatory/inhibitory balance and center/surround organization in the granular layer.

(A) VSDi normalized maps showing the spatial distribution of excitation and inhibition in WT and IB2 KO granular layer (average of 5 recordings in both cases).

(B) The plot shows the E/I balance as a function of distance from the center for the maps shown in A. Note that in IB2 KO granular layer the excitation core is broader, while the inhibited surround is reduced, compared to WT. This tends to change the C/S from the typical Mexican hat in control to stovepipe hat shape in IB2 KO mice (cf. inset).

(C) The histograms show, in WT and IB2 KO mice, the average values of the inhibition or excitation areas and of core diameter. Data are reported as mean ± SEM;; *p<0.05, **p<0.01.

Figure 4. NMDA receptor-dependent component of granular layer excitation.

(A) VSDi normalized maps showing the spatial distribution of the NMDA component of excitation in WT and IB2 KO granular layer (average of 5 recordings in both cases).
(B) The plot shows the NMDA component as a function of distance from the center for the maps shown in A. Note that in IB2 KO granular layer the NMDA component of excitation is larger and more extended compared to WT.

(C) The histograms show, in WT and IB2 KO mice, the average values of the NMDA component normalized amplitude and of core diameter. Data are reported as mean ± SEM; **p<0.01.

**Figure 5. LTP of mossy fiber-granule cell EPSCs.**

(A) Granule cell synaptic responsiveness. Voltage responses were elicited from -50 mV during 1 sec-100 Hz synaptic stimulation (HFS) used for plasticity induction. Note stronger spike generation in IB2 KO than WT.

(B) The histogram shows the average number of spikes during HFS in WT and IB2 KO mice. Data are reported as mean ± SEM; *p<0.05.

(C) Long-lasting effects of HFS on EPSC\_AMPA in WT and IB2 KO granule cells. EPSC\_AMPA (average of 30 tracings in both cases) are recorded in control and 20 min after HFS. Note that, after HFS stimulation, the EPSC\_AMPA increase was larger in IB2 KO than WT. The LTP plot shows the average time course of EPSC\_AMPA amplitude changes in WT (n=12) and IB2 KO (n=9) granule cells. Data are reported as mean ± SEM; *p<0.05.

(D) The histogram shows the average EPSC\_AMPA LTP following HFS in WT and IB2 KO mice. Data are reported as mean ± SEM; *p<0.05.

**Figure 6. Long-term enhancement in granule cells intrinsic excitability.**

(A) Voltage responses to current injection in WT and IB2 KO granule cells recorded in control and 20 min after HFS. Note that HFS enhances spike generation both in WT and IB2 KO granule cells.

(B) Spike frequency is plotted as a function of current injection in control conditions and after HFS both in WT and IB2 KO mice. Note that, after HFS, spike frequency increases more in WT than in
IB2 KO mice. The histograms compare the average spike frequency and threshold current (I\text{th}) changes in WT and IB2 KO mice. Data are reported as mean ± SEM; *p<0.05, **p<0.01.

(C) Subthreshold voltage responses to current injection in WT and IB2 KO granule cells recorded in control and 20 min after HFS. Note that the voltage-response in the high-potential region is enhanced both in WT and IB2 KO granule cells.

(D) The plot shows the average time course of input resistance (R\text{in}) changes after HFS stimulation in two subthreshold membrane potential regions, < -70 mV (R\text{in-low}) and > -70 mV (R\text{in-high}). After HFS, in both WT and IB2 KO granule cells, R\text{in-high} but not R\text{in-low} increased. The histogram shows the average R\text{in} changes for WT and IB2 KO mice. Data are reported as mean ± SEM; **p<0.01.

Figure 7. NMDA and GABA\textsubscript{A} receptor dependence of LTP

(A) Long-lasting effects of HFS on EPSC\textsubscript{AMPA} in WT and IB2 KO granule cells during blockade of NMDA receptors with D-APV (50 μM) and 7-Cl-Kyn acid (50 μM). EPSC\textsubscript{AMPA} (average of 30 tracings in both cases) are recorded in control and 20 min after HFS. Note that, after HFS stimulation, IB2 KO cells show LTD, while WT cells do not show any significant changes. The plot shows the average time course of EPSC\textsubscript{AMPA} amplitude changes in WT (n=7) and IB2 KO (n=6) granule cells. Data are reported as mean ± SEM.

(B) The histogram shows the average EPSC\textsubscript{AMPA} change following HFS in WT and IB2 KO mice. Data are reported as mean ± SEM; *p<0.05.

(C) Long-lasting effects of HFS on EPSC\textsubscript{AMPA} in WT and IB2 KO granule cells in gabazine-free extracellular solution. EPSC\textsubscript{AMPA} (average of 30 tracings in both cases) are recorded in control and 20 min after HFS. Note that, after HFS stimulation, the LTP observed in control is no longer visible and is substituted by an EPSC\textsubscript{AMPA} LTD in IB2 KO mice that is larger than WT mice. The plot shows the average time course of EPSC\textsubscript{AMPA} amplitude changes in WT (n=6) and IB2 KO (n=7) granule cells. Data are reported as mean ± SEM.)
Figure 8. Mechanisms of LTP expression.

(A) EPSC\textsubscript{AMPA} in WT and IB2 KO granule cells (average of 30 tracings in both cases) recorded in control and 20 min after HFS using paired-pulse stimulation (interstimulus interval 20 ms). The histogram shows the CV, PPR and EPSC\textsubscript{AMPA} amplitude changes following HFS in WT and IB2 KO mice. Data are reported as mean ± SEM; \*p<0.05, **p<0.01.

(B) The \((CV_2/CV_1)^2\) vs. \((EPSC_2/EPSC_1)\) plot shows that WT LTP points fall in the sector of increased quantal release (>\(p,n\)) while IB2 KO points fall on the diagonal (>\(n\)) and in the sector of increased quantum size (>\(q\)).

(C) The traces show spontaneous synaptic activity before and after LTP induction in WT and IB2 KO granule cells. Following LTP induction, mEPSC frequency, but not amplitude, increased in WT while mEPSC amplitude, but not frequency, increased in IB2 KO mice.

(D) Examples of individual mEPSCs before and after LTP induction in WT and IB2 KO granule cells. The histograms compare changes in mEPSC frequency and amplitude during LTP in WT and IB2 KO mice. Data are reported as mean ± SEM; *p<0.05.

Figure 9. Spatial distribution of long-term plasticity of granular layer responses to mossy fiber stimulation.

(A) VSDi normalized maps showing the spatial distribution of LTP and LTD in WT and IB2 KO granular layers (average of 6 recordings in both cases).

(B) The plot shows plasticity as a function of distance from the center for the maps shown in A. Note that in IB2 KO the LTP magnitude in the core is larger, and that the core is broader than in WT.
(C) The histograms show, in WT and IB2 KO mice, the average core diameter and the LTP and LTD amplitude 30 minutes after HFS (n=6 for both). Note that the IB2 KO granular layer shows larger LTP smaller LTD and larger cores than WT. Data are reported as mean ± SEM; ***p<0.001; **p<0.01.

(D) VSDi recordings showing LTP and LTD of granular layer responses to mossy fiber stimulation. Exemplar traces before and 30 minutes after the induction protocol are reported for WT and IB2 KO. The plot shows the average time course of LTP and LTD for WT and IB2 KO (n=6 for both). Data are reported as mean ± SEM.

References


Table 1. Properties of whole-cell recordings in mice granule cells

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=92)</td>
<td>(n=76)</td>
</tr>
<tr>
<td>$R_m$ (GΩ)</td>
<td>2.0 ± 0.1</td>
<td>2.7 ± 0.2 *</td>
</tr>
<tr>
<td>$C_m$ (pF)</td>
<td>3.6 ± 0.1</td>
<td>3.2 ± 0.1 ***</td>
</tr>
<tr>
<td>$R_s$ (MΩ)</td>
<td>17.6 ± 1.1</td>
<td>18.3 ± 1.0</td>
</tr>
<tr>
<td>$f_{VC}$ (KHz)</td>
<td>3.5 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>$V_m$ (mV)</td>
<td>-50.2 ± 1.4 (n=34)</td>
<td>-49.2 ± 1.5 (n=30)</td>
</tr>
</tbody>
</table>
A

IPSC

WT
KO

EPSC (AMPA)

10 pA
5 ms

EPSC (NMDA)

20 pA
25 ms

B

IPSC amplitude (pA)

EPSC\textsubscript{AMPA} amplitude (pA)

WT
KO

EPSC\textsubscript{AMPA}/IPSC ratio

EPSC\textsubscript{NMDA}/IPSC ratio

**