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REVIEW ESSAY

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Plasticity mechanisms of genetically distinct Purkinje cells

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Abstract

Despite its uniform appearance, the cerebellar cortex is highly heterogeneous in terms of structure, genetics and physiology. Purkinje cells (PCs), the principal and sole output neurons of the cerebellar cortex, can be categorized into multiple populations that differentially express molecular markers and display distinctive physiological features. Such features include action potential rate, but also their propensity for synaptic and intrinsic plasticity. However, the precise molecular and genetic factors that correlate with the differential physiological properties of PCs remain elusive. In this article, we provide a detailed overview of the cellular mechanisms that regulate PC activity and plasticity. We further perform a pathway analysis to highlight how molecular characteristics of specific PC populations may influence their physiology and plasticity mechanisms.

KEYWORDS
differential gene expression, intrinsic excitability, intrinsic plasticity, long-term plasticity, Purkinje cell, synaptic plasticity, Zebrin-II

INTRODUCTION

Modulation of Purkinje cell activity

The cerebellum is a brain structure essential for motor control, motor learning and sensorimotor adaptation.[1,2] Due to the continuously changing demands posed by a changing environment, the cerebellum must be endowed with a considerable capacity for learning. Central to cerebellar learning is the modulation of Purkinje cell (PC) activity.[3–6] PCs are the sole output neuron of the cerebellar cortex innervating the cerebellar nuclei (CN) (Figure 1A). The CN, in turn, project to downstream brain areas, some of which directly control muscles.[7] Changes

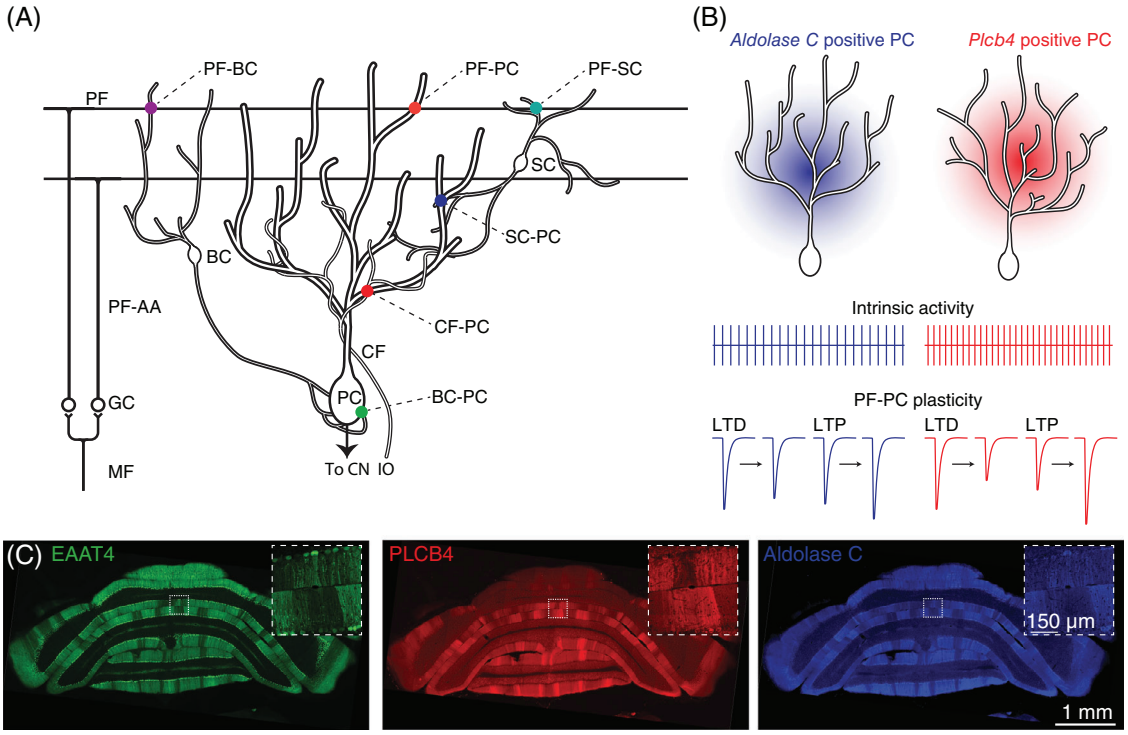

FIGURE 1 The cerebellar circuit and the basic characteristics of *Aldoc*+ and *Plcb4*+ PCs. (A) Simplified overview of the cerebellar circuit with a Purkinje cell (PC) receiving inputs from parallel fibers (PF), a single climbing fiber (CF) originating from the inferior olive (IO), basket cell (BC), and stellate cell (SC). Mossy fibers provide inputs to granule cells which give rise to PFs. Important synapses are highlighted with a circle and dashed line. (B) Two distinct subpopulations of PCs are highlighted with blue (*Aldolase C* positive) and red (*Plcb4* positive). *Plcb4* positive PCs have a higher intrinsic action potential rate and an increased propensity for long term plasticity, namely, long term synaptic potentiation (LTP) and long term depression (LTD). (C) The expression of EAAT4 (left), PLCB4 (Middle), and Aldolase C (right) in a coronal section of the cerebellum. Insets, magnification of lobule VII/VIII. EAAT4 and Aldolase C are expressed in the same pattern, whereas *Plcb4* is expressed in a complementary pattern. Images adapted from ref. [28]. The images from Figure 1c were adapted from ref. [28], an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

in PC activity therefore impacts CN activity and ultimately the output of the cerebellum.[8,9]

An important mechanism that modulates PC activity is synaptic plasticity at the parallel fiber (PF) to Purkinje cell (PC) synapse.[10] Cerebellar granule cells (GCs), which give rise to PFs, provide the most numerous excitatory synaptic connection to PCs (Figure 1A). In rats, each PC can receive upwards of 200,000 PF-PC synapses,[11] and this number drastically increases in humans.[12] As such, PF activity is critical in shaping the rate and temporal pattern of action potentials in PCs (Figure 1A). GCs are excited by mossy fibers (MFs), which carry sensory information from brain areas such as the pontine nuclei, vestibular nuclei, and the spinal cord (Figure 1A).[13] MF activation of GCs therefore indirectly leads to increased activity in PCs and reduced activity in CN neurons.[14] Coincident activation of PFs with yet another type of excitatory input, climbing fibers (CF, CF-PC synapse, Figure 1A) originating in the inferior olive (IO), induces long term changes in the synaptic efficacy of the PF-PC synapse.[15,16] Thus, the information carried by PF inputs is altered by specific combinations of synaptic inputs from the PFs and CF.

Plasticity at the PF-PC synapse is the most studied form of synaptic plasticity in the cerebellum and has been correlated with several synaptic as well as intrinsic mechanisms.[17–20] PF-PC synapses can become stronger, measured as an increase in the synaptic response elicited by PF activation, a process that is called long-term potentiation (LTP). Conversely, PF-PC synapses can also become weaker, a process called long-term depression (LTD).[10,15,21] PF-PC synapses are strengthened by repeated PF activity in the absence of CF signals,[21–23] while PF-PC synapses are weakened when PF signals coincide with CF signals within strict timing intervals.[15,23,24] LTD reduces the strength of the excitatory inputs to PCs, whereas LTP increases them, both of which influence the generation of action potentials.

Other mechanisms may also influence PC firing, such as plasticity at the molecular layer interneuron (MLI)-PC synapse, and plasticity of the intrinsic excitability (IE) of PCs (long-term potentiation of intrinsic excitability; LTP-IE, long-term depression of intrinsic excitability; LTD-IE).[19,25,26] MLIs, namely, basket (BC) and stellate (SC) cells provide feed-forward inhibition to PCs, and receive inputs from PFs. (Figure 1A). These plasticity mechanisms may occur simultaneously, for example, depression of the PF-PC synapse is also known to depress the intrinsic activity of PCs.[27] Thus, through specific inputs, different mechanisms at different synapses shape the activity of a PC, and thus the output of the cerebellar cortex.

MAIN TEXT

Relation between molecular diversity and physiological properties of Purkinje cells

Regional heterogeneity of cell types is an important aspect of cerebellar function (Figure 1B).[7,28–33] PCs with unique molecular profiles can be distinguished and aggregate together to form bands or "stripes" (Figure 1B,C).[34] These bands are highly organized, each receiving CF and PF inputs from specific regions of the inferior olive and pontine nuclei, and projecting to specific regions of the CN (for review see ref. [35]). These unique PCs may be identified by their expression of specific molecular markers, such as *Aldolase C*. However, besides their levels of *Aldolase C* expression, PCs can also be identified by their levels of phospholipase C beta 4 (*Plcb4)* expression, which follows an opposite expression pattern relative to *Aldolase C* (Figure 1C).[36] Many other molecules share a similar expression pattern, such as the excitatory amino acid transporter 4 (EAAT4, Figure 1C), phospholipase C beta 3 (Plcb3), and metabotropic glutamate receptor 1b (mGluR1b).[37–39] The development of these distinct molecular profiles is tightly regulated from early brain development.[40] An important regulator of a PCs molecular profile is the transcription factor *Ebf2*. *Ebf2* actively suppresses the *Aldolase C* phenotype, and thus *Ebf2*+ PCs develop into PCs expressing *Plcb4*, whereas PCs which lack *Ebf2* expression develop into PCs expressing *Aldolase C*.[41] More recently, several publications employing single-cell RNA sequencing revealed a further 400+ genes, which may be roughly divided into two groups which correlate with the expression of either *Aldolase C* or *Plcb4* (Figure 1B).[42–45]

PCs located in different bands are known to have distinctive physiological properties (Figure 1B).[32,46,47] PCs in bands expressing low levels of *Aldolase C* and high levels of *Plcb4* (*Plcb4*+) have a higher intrinsic SS activity (~90 Hz vs. ~60 Hz) and an increased CS frequency (~1.3 Hz vs. ~1.0 Hz), whereas PCs in bands expressing high levels of *Aldolase C* but low levels of *Plcb4* (*Aldoc*+) have complex spikes with an increased number of spikelets and a larger charge transfer (Figure 1B).[32,46,48] Additionally, PCs located in *Aldoc*+ versus *Plcb4*+ bands have distinct propensities for postsynaptic as well as intrinsic plasticity.[28,29,31,49] *Plcb4*+ PCs have a higher propensity for PF-PC LTD compared to *Aldoc*+ PCs, meaning that the same induction protocols lead to a larger reduction in synaptic strength (Figure 1B).[28,29] The differences in LTP are more ambiguous, as inducing LTP with low frequency (1 Hz) PF stimulation results in increased LTP in *Plcb4*+ PCs,[31] while stimulation at higher frequencies (8 at 100 Hz) does not.[28] In this review, we focus our discussion on postsynaptic plasticity at the PF-PC synapse, and plasticity of intrinsic excitability.

The precise molecular mechanisms facilitating PF-PC plasticity are relatively well-characterized (Figure 2A, B; based on refs. [10, 50, 51]). The primary mechanism of plasticity at the PF-PC synapse involves glutamate release at the PF-PC synapse, followed by the activation of ionotropic glutamate receptors, predominantly α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Both LTP and LTD are characterized by changes in the conductivity or trafficking of AMPA receptors.[50,52–54] During LTP, increased conductance and/or expression of AMPA receptors serves to increase the chance of eliciting an action potential.[55] During LTD, the internalization of AMPA receptors from the postsynaptic membrane reduces the chance of eliciting an action potential.[56] Glutamate release also activates metabotropic glutamate receptors (mGluRs), mainly mGluR1. mGluR1 activates phospholipase C beta (PLCB), which catalyzes the production of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 is an important messenger molecule which triggers calcium release from the endoplasmic reticulum (ER).[57] Increased intracellular calcium concentrations lead to the activation of several second-messenger cascades. Calcium activates protein kinase C (PKC), which internalizes AMPA receptors, leading to LTD.[53,56,58,59] Calcium also activates protein phosphatase 2B (PP2B), which promotes increased conductance of AMPA receptors, or the addition of AMPA receptor to the postsynaptic membrane, leading to LTP.[55,60–62] The amount of intracellular calcium decides whether LTP or LTD is induced.[23,50,63] Small increases in intracellular calcium will induce LTP,[23] but once a threshold-exceeding amount of calcium is reached, self-sustaining processes will instead promote the induction of LTD. This threshold is regulated by calcium/calmodulin-dependent protein kinase II (CaMKII), which negatively regulates the activity of protein phosphatases, and requires a large amount of free calcium to be activated.[56,63–65] Large amounts of calcium release is facilitated by CF activity.[23] Several "supporting" signals also play a role in the induction of LTP and/or LTD, such as endocannabinoid or nitrous oxide (NO) signaling.[21,50,54,62] In the following sections we will discuss in more detail the principle cellular mechanisms underlying postsynaptic plasticity at the PF-PC synapse and discuss their relation to *Aldoc* and *Plcb4* expression in PCs.

Role of ionotropic glutamate receptors in plasticity

Glutamate release and the baseline AMPA-induced currents at PF-PC synapses are known to be comparable between *Aldoc*+ versus *Plcb4*+ PCs.[28,66] However, the subunit composition of AMPA receptors may differ. This composition is important, because it determines how the receptor interacts with other intracellular factors, such as those involved in the induction of synaptic plasticity.[67,68] AMPA receptors consist of a combination of four subunits, encoded by four genes, GluA1-4. AMPA receptors of PCs typically contain the GluA2 subunit.[69] GluA2 plays a key role in several mechanisms regulating plasticity, such as the ability of the receptor to bind protein kinase C (PKC) and N-ethylmaleimide-sensitive factor (NSF).[67,68,70,71] GluA2 also renders the AMPA receptor impermeable to calcium.[72] *Plcb4*+ PCs express higher levels of the GluA2 subunit, whereas *Aldoc*+ PCs express higher levels of GluA1.[42,44] Thus, the AMPA receptors of Plcb4+ PCs may have a higher predisposition for being trafficked by molecules such as PKC or NSF, favoring both LTD and LTP.

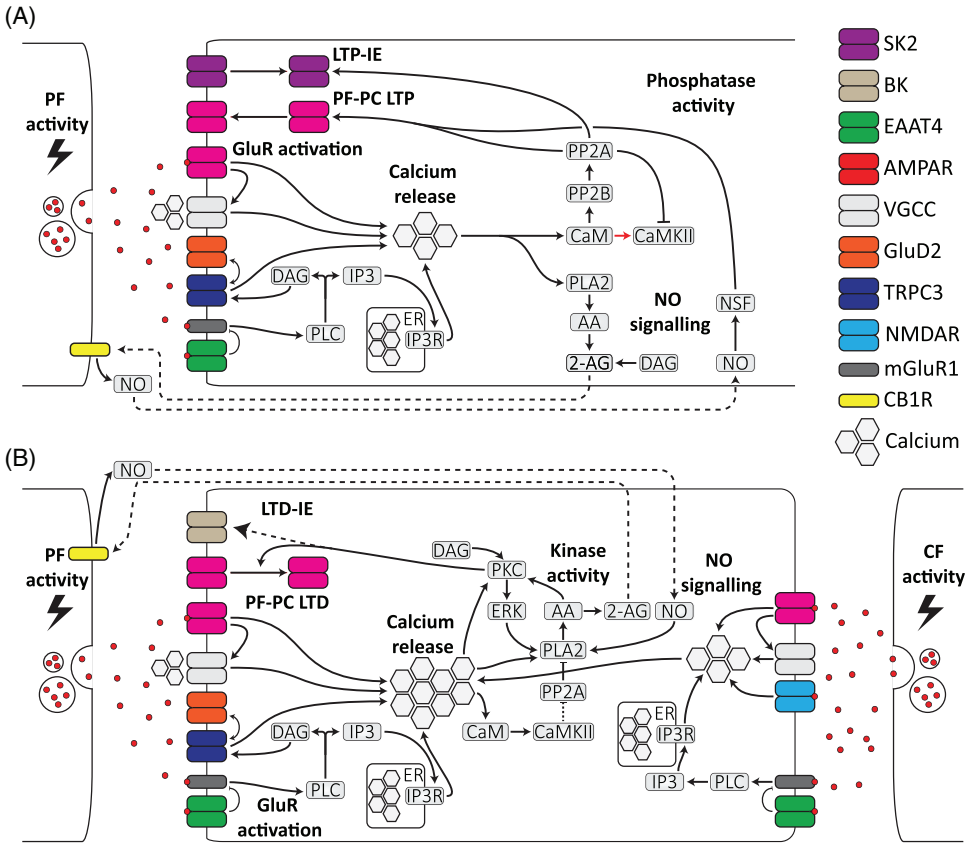

FIGURE 2 Molecular mechanisms of synaptic and intrinsic plasticity in PCs. (A,B) Molecular mechanisms of LTP/LTP-IE (A) and LTD/LTD-IEi. (B) The activation of glutamate receptors triggers calcium release from several channels and internal stores. Calcium activates downstream targets like CaM, PKC, PLA2, and PP2A. PP2A facilitates the induction of LTP and LTP-IE by addition of AMPA receptors or removal of SK2 channels. PKC facilitates the induction of LTD and possibly LTD-IE by removing AMPA receptors and modifying BK channels, respectively. PKC activity is maintained by the activation of CaMKII which triggers a positive feedback loop. Nitrous oxide signaling via PLA2 → 2-AG → CB1R → NO can support either the activation of PKC by the production of AA, or support LTP by the activation of NSF. Similar to PP2A, NSF facilitates the addition of AMPA receptors. Mechanisms are based on refs. [10, 50].

Another important type of ionotropic glutamate receptor expressed by cerebellar PCs in adult mice is the *N–Methyl–D–aspartate* (NMDA) receptor. PCs express NMDA receptors containing the GluN2 subunit,[73,74] which are postsynaptically localized at CF-PC synapses. NMDA receptors contribute significantly to the calcium signal triggered by CF activity (Figure 2).[73,74] The activation of presynaptic NMDA receptors on PFs also plays a role in the induction of both LTP and LTD.[75] NMDA receptors contribute to LTD and LTP by stimulating presynaptic nitrous oxide (NO) production.[76] NO contributes to LTD through activation of phospholipase A2 (PLA2), and the production of arachidonic acid (AA), whereas it contributes to LTP through activation of NSF (Figure 2).

Plcb4+ PCs specifically express NMDA receptor subunit GluN2a, whereas expression is almost zero in *Aldoc*+ PCs (Figure 3D–F).[44] Although physiological evidence for differences in NMDA currents in *Aldoc*+ versus *Plcb4*+ is lacking, one may predict that differences in GluN2a expression influences CF-evoked calcium currents. Therefore, increased NMDA receptor expression in *Plcb4*+ PCs may contribute to the increased propensity for synaptic plasticity observed in *Plcb4*+ PCs.

Role of metabotropic glutamate receptors in plasticity

Unlike AMPA receptors, metabotropic glutamate receptors (mGluRs) influence cellular processes through second messengers. mGluR1 signaling plays a key role in the signaling cascade of PF-PC plasticity through its downstream targets.[77–81] Activation of mGluR1 triggers PLCB activity, which facilitates the production of IP3 and DAG (Figure 2). This triggers calcium release from the ER by IP3, and from the extracellular space through the activation of GluD2 and TRPC3 by DAG (Figure 2).[57,82] mGluR1 activation also plays a role in the activation of PKA, which plays a role in the induction of PF-PC plasticity, as well as intrinsic plasticity (Figure 4F).[50,51] The mGluR1 signaling pathway contains many molecules which are specifically enriched in either *Plcb4*+ and *Aldoc*+ PCs.[39,42,44] The mGluR1 (GRM1) receptor itself shows slightly increased expression in the Plcb4+ population (Figure 3D–F). This is represented by increased expression of the specific mGluR1b splice variant.[39]

PF stimulation results in enhanced PF-mGluR1 postsynaptic currents (EPSC) in *Plcb4*+ PCs compared to *Aldoc*+ PCs.[49] The

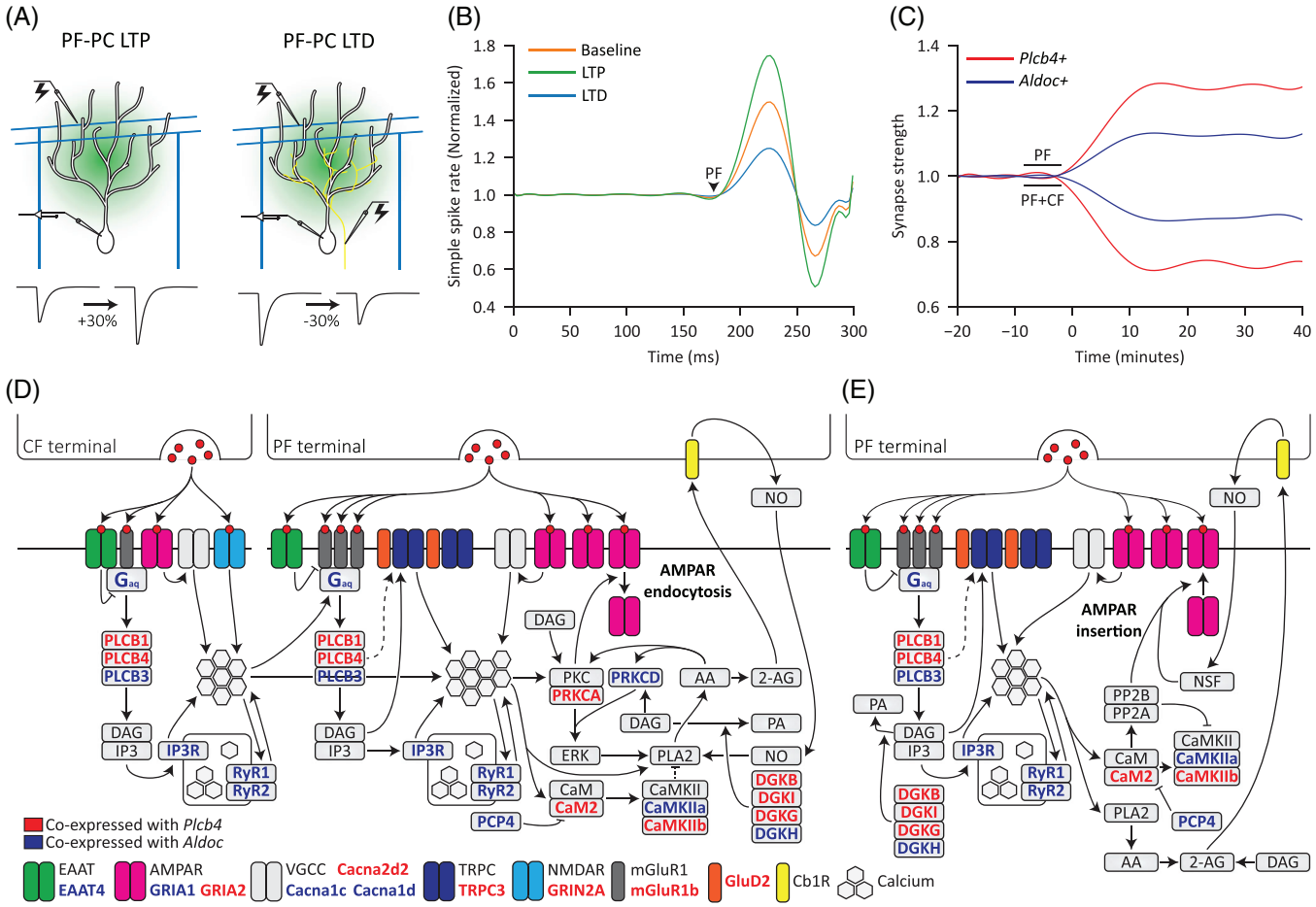

FIGURE 3 Parallel fiber to Purkinje cell plasticity in *Aldoc*+ and *Plcb4*+ PCs. (A) Schematic representation of a typical long term potentiation (LTP, left) and long term depression (LTD, right) experiment. (B) Schematic concept of the effects of LTP and LTD on simple spike rate of PCs. LTP increases simple spike rate more so than baseline conditions upon PF stimulation, whereas LTD reduces the increase in simple spike rate compared to baseline conditions. (C) Schematic illustrating the induction of LTP and LTD in PCs with specific molecular identities. Changes in the synaptic strength of PF-PC synapses are different for *Aldoc*+ versus *Plcb4*+ PCs. Illustration is based on data from refs. [28, 29, 31]. (D, E) Molecular mechanism of LTD (D) and LTP (E), with molecular components highlighted based on their expression in *Aldoc*+ (blue) or *Plcb4*+ (red) PCs. Dashed lines indicate an indirect connection with unknown intermediates.

PF-mGluR1 EPSC is mediated primarily by the transient receptor potential channel 3 (TRPC3). TRPC3 currents are enhanced by intradendritic calcium, such as from coincident CF activation during LTD induction.[29,83–85] Many of the molecular components involved in the mGluR1-TRPC3 pathway are enriched in *Plcb4*+ PCs. These include the actual cation channel, TRPC3, and many proteins that interact with TRPC3 such as STIM1, GRID2, and Homer3 (Figures 3D, E and 4E, F).[42,44,86]

Metabotropic glutamate receptors regulate plasticity through activation of second-messenger cascades

A key component of mGluR1s capacity for activating its downstream targets is its associated G-protein(s). In PCs, certain G-proteins show selectively enriched expression in either *Aldoc*+ or *Plcb4*+ PCs. *Aldoc*+ PCs show enhanced expression of the alpha (GDP-GTP converting) subunits GNAO1, GNAL, and GNAQ. *Plcb4*+ PCs show enhanced expression of GNAI1.[44] Of particular interest is GNAQ, which belongs to the G-protein q/11 family, whose members facilitate the activation of PLCB (Figures 3D, E and 4E, F).[87] The expression of PLCB also shows considerable heterogeneity within PC subpopulations. The beta variant of PLC is commonly expressed within PCs, especially *Plcb*1, 3, and 4. However, *Plcb*1 and 4 are highly expressed in the *Plcb4*+ population, whereas *Plcb3* is highly expressed in the *Aldoc*+ population (Figures 3D, E and 4E, F).[38,44] The functional differences between these variants of *Plcb* are still unclear, although it is evident that there are different variants of this similar protein that are involved at this stage of the mGluR1 signaling pathway.

Glutamate transporters play a key role in neuronal function and plasticity

Excitatory amino acid transporters are important regulators of synaptic function. Glutamate transporters play an essential role in main-

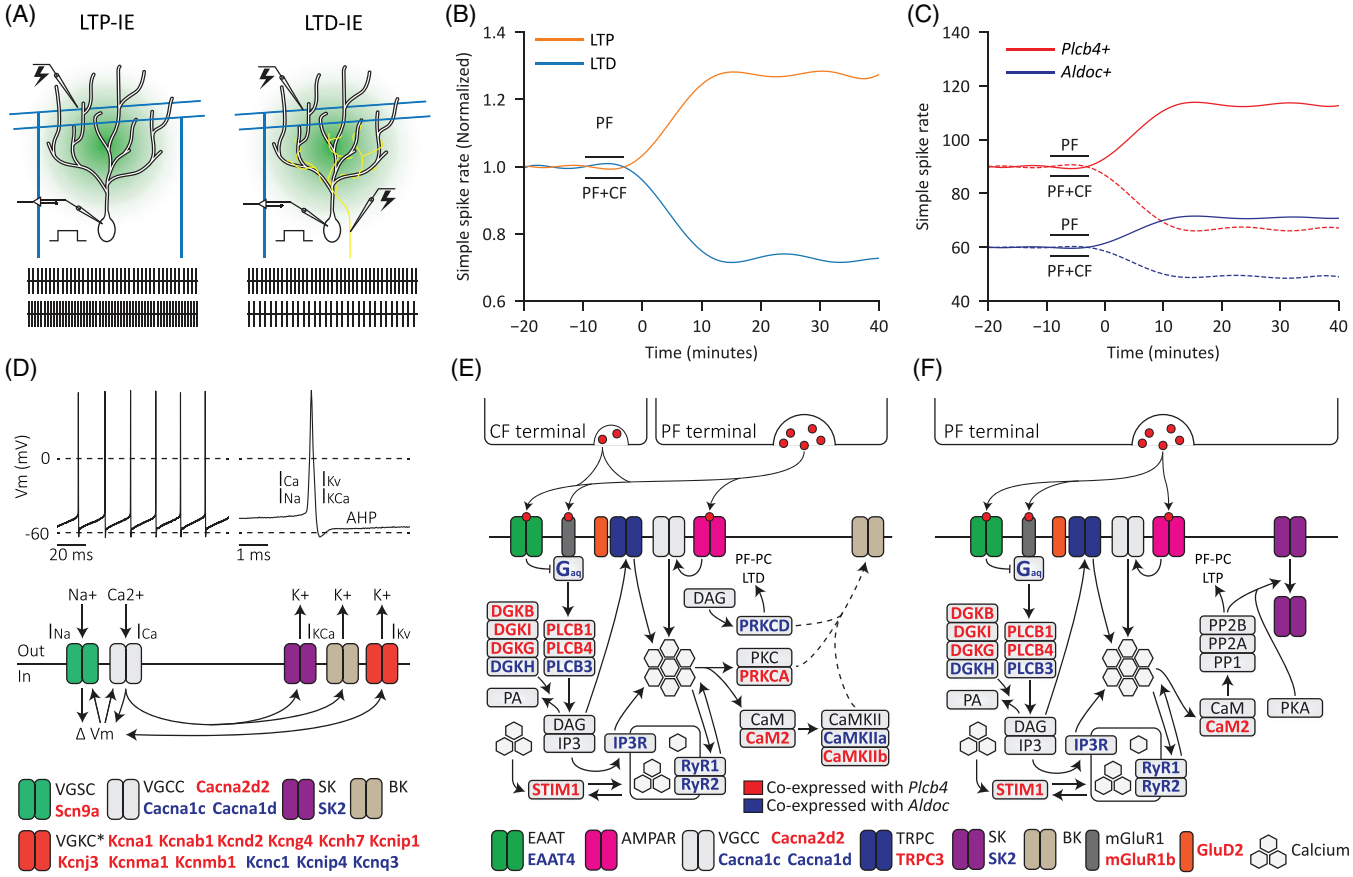

FIGURE 4 Intrinsic activity and plasticity of intrinsic activity in *Aldoc*+ and *Plcb4*+ PCs. (A) Schematic representation of an induction protocol used for the induction of LTP of intrinsic excitability (left) and LTD of intrinsic excitability (right). (B) Schematic illustrating the effect of LTP-IE (orange) and LTD-IE (blue) on simple spike activity in PCs. LTP-IE facilitates a long term increase in simple spike activity independent of synaptic activity, whereas LTD-IE facilitates a long term decrease in simple spike activity. (C) Schematic illustrating the baseline simple spike rate and the induction of LTP-IE and LTD-IE in PCs with specific molecular identities. LTP-IE increases simple spike rate more in *Plcb4*+ PCs. We predict that the same is true for LTD-IE but this has not been shown experimentally, and thus is represented by a dashed line. LTP-IE data are based on ref. [31]. (D) Spontaneous action potentials recorded from a PC in current clamp mode with no injected bias currents (top left) and an individual spike and the associated ionic currents (top right). Depolarization is facilitated by sodium (I_{Na}) and calcium currents (I_{Ca}), whereas repolarization and after hyperpolarization (AHP) is facilitated by voltage gated potassium- (K_V)and calcium activated potassium (K_{Ca}) currents (top right, bottom). Ion channels enriched in *Aldoc*+ or *Plcb4*+ PCs are highlighted in blue or red, respectively. (E, F) Molecular mechanism of LTD-IE (E) and LTP-IE (F), with molecular components highlighted based on their expression in *Aldoc*+ (blue) or *Plcb4*+ (red) PCs. Dashed lines indicate an indirect connection with unknown intermediates. Illustration based on ref. [51].

taining proper neuronal function, protecting neurons from the toxic effects of glutamate overexposure through rapid removal, and limiting the action of glutamate during synaptic transmission.[88,89] Within the cerebellum, glutamate released from PFs and CFs is transported by glutamate transporters expressed in PCs and Bergmann glia. PCs express both EAAT3, and EAAT4, whereas Bergmann glia express EAAT1.[37,90,91]

The expression of the transporter EAAT4 is enriched in *Aldoc*+ PCs, and has a strong correlation with the expression of *Aldolase C* (Figures 3D, E and 4E, F). EAAT4 limits the activation of mGluR1, and thus plays a role in PF-PC plasticity.[29] Both EAAT4 and mGluR1 are commonly expressed in the peri-synaptic zone.[91–94] Reduced expression of EAAT4 in *Plcb4*+ PCs may thus lead to increased activation of the mGluR1 pathway, critical for the induction of plasticity, whereas increased EAAT4 expression in *Aldoc*+ PCs reduces the overall activity in the mGluR1 pathway. Altogether, reduced EAAT4 expression alongside increased mGluR1(b) expression may serve to synergistically increase TRPC3/IP3 mediated calcium influx in *Plcb4*+ PCs. In *Aldoc*+ PCs, high EAAT4 expression may instead limit the activation of relatively few mGluR1 receptors, further reducing the total activation of the mGluR1 pathway, and thus LTD and LTP.

Calcium is a crucial mediator of plasticity at the parallel fiber—Purkinje cell synapse

Calcium is a crucial second messenger regulating the induction of both plasticity and intrinsic activity (Figure 2). Calcium is an activator of calmodulin (CaM), which itself activates CaMKII and PP2B, necessary for LTD and LTP, respectively (Figure 2).[60,64,95] The calcium dynamics

of *Aldoc*+ and *Plcb4*+ PCs are thus critical determinants of the direction and degree of plasticity. Many receptors contribute to increasing intracellular calcium concentrations during plasticity, including mGluR1, TRPC3, IP3 receptors, Ryanodine receptors, AMPA receptors, and NMDA receptors. The total calcium influx is also regulated by the activation of voltage gated calcium channels (VGCCs).

Aldoc+ and *Plcb4*+ PCs differentially express several VGCC subunits (Figures 3D, E and 4E, F). For example, there is increased expression of the auxiliary subunit *Cacna2d2* in *Plcb4*+ PCs. *Cacna2d2* plays an important role in the modulation of calcium currents. *Cacna2d2* knockouts show increased glutamate release, and glutamate clearance at CF-PC synapses, which reduces the overall number of spikelets, but increases the overall charge transfer at CF-PC synapses.[96] In *Aldoc*+ PCs, where cacna2d2 expression is relatively low, there is evidence for enhanced glutamate release at CF-PC synapses, alongside larger synaptic charge transfer, similar to what is observed in *Cacna2d2* knockouts.[48]

Expression of calcium channels in different Purkinje cell populations

Cacna1c (Cav1.2) and *cacna1d* (Cav1.3) are enriched in *Aldoc*+ PCs (Figures 3D, E and 4E, F).[44] These two channels are both L-type calcium channels and are known to regulate neuronal excitability, and synaptic plasticity in the hippocampus and amygdala.[97–99] In the cerebellum, Cav1.3 knockout mice show impaired motor- and associative learning.[100] *Aldoc*+ PCs also have increased expression of the calcium channels on the endoplasmic reticulum, the Ryanodine receptors *RyR1* and *RyR2*, and the IP3 receptor *ITPR3*.[44,101] RyRs are calcium sensing channels, releasing calcium in response to calcium. *ITPR3* releases calcium when IP3 is bound, and IP3 is a product created by the activation of Plcb. There may also be a functional interaction between *Cacna1c* and the ryanodine receptors *RyR1* and *RyR2*.[102] Based on the expression of RyRs and *ITPR3*, there appears to be an increased calcium release from the endoplasmic reticulum in *Aldoc*+ PCs, which has also been observed experimentally.[49] High-frequency PF stimulation triggers calcium release in parasagittal bands which align with *Aldoc*+ bands, but not *Plcb4*+ bands. This release can be blocked by blocking mGluR1, Plcb, or RyRs. The release of calcium from the ER may thus be, at least in response to PF input, different between *Aldoc*+ and *Plcb4*+ PCs.

Stromal interaction molecule 1 (STIM1) is also an important regulator of intracellular calcium, and is enriched in *Plcb4*+ PCs. STIM1 regulates proper calcium influx after mGluR1 signaling, and motor coordination.[103] STIM1 functions by regulating the intracellular calcium concentration, as it is responsible for refilling stored calcium in the ER. Although not implicated in synaptic plasticity, STIM1 is essential in controlling intrinsic excitability, and intrinsic plasticity.[104] STIM1 also regulates TRPC3.[103,104] TRPC3 knock-out mice show altered intrinsic activity, but this predominantly affects *Plcb4*+ PC.[86] Enhanced expression of STIM1 in *Plcb4*+ PCs may facilitate more efficient uptake of calcium during intrinsic activity (Figures 3D, E and 4E, F). This could, for example, limit the activation of calcium activated K+ channels, which are important regulators of neuronal excitability.[105]

Calcium-activated proteins mediate plasticity and are differentially expressed in Purkinje cell populations

Calmodulin (CaM) plays an important role in the induction of LTD, through the activation of CaMKII, but also LTP, by activating PP2B. CaM is activated by calcium. Whether LTP or LTD is induced depends on the degree of CaM activation, and thus the calcium concentration. The CaM variant *CaM2* is specifically enriched in *Plcb4*+ PCs.[44] The activation of CaM is also regulated by a protein called Purkinje cell protein 4 (pcp4). Pcp4 negatively regulates the binding of calcium to CaM, and also plays an important role in the induction of PF-PC plasticity. Similar to mutations in CaMKII, mice lacking Pcp4 inverse their direction of plasticity; activity that normally induces LTD now induces LTP, and vice versa.[106] Notably, Pcp4 is highly expressed in *Aldoc*+ PCs (Figures 3D, E and 4E, F). Limited activation of CaM in these subpopulations may further limit the activation of both PP2B and CaMKII, and thus influencing the direction of plasticity.

CaM is an important activator of Calcium/Calmodulin dependent kinase II (CaMKII). CaMKII is a holoenzyme consisting of several isoenzymes. In PCs, isoenzymes of alphaCaMKII and betaCaMKII are most common, occurring at roughly a 1:1 (a:b) ratio.[107] Each of these subunits have specific functions. Genetic knockout of CaMKIIa in PCs abolishes the induction of LTD.[95] Similar to Pcp4 knockouts, CaMKIIb knockout show reversed directions of plasticity, indicating that CaMKIIb is especially important in controlling the direction of synaptic plasticity.[64,108] Activated CaMKII disinhibits a positive feedback loop involving ERK, PLA2, AA, and PKC.[56,65] However, if CaMKII fails to activate, the activity of PP2B (and other phosphatases) prevails, essentially preventing the induction of LTD.[50]

CaMKII subunits are differentially expressed in *Aldoc*+ and *Plcb4*+ PCs (Figures 3D, E and 4E, F). A striped expression pattern has been identified for CaMKIIa, although it only partially overlaps with the expression of *Aldolase C*.[109] CaMKIIb may be enriched in *Plcb4*+ PCs, as shown by single cell RNA sequencing.[44] In the hippocampus, neuronal activity regulates the ratio between alpha and beta subunits, where higher activity increases CaMKIIa, and lower activity increases CaMKIIb.[110] The opposite is seen in PCs, where *Plcb4*+ PCs have higher baseline activity, and express higher levels of CaMKIIb.[32] *Aldoc*+ PCs have lower baseline activity, and express a larger fraction of CaMKIIa.[32] Variations in the ratio between alpha and beta CaMKII may thus be an important mechanism regulating the differential propensity for plasticity.

Protein kinase C (PKC) is critical in facilitating LTD at the PF-PC synapse, as it is responsible for the phosphorylation of AMPA receptors, and thus their removal from the postsynaptic membrane (Figure 2).[56,111] Sustained PKC activity is essential for PF-PC LTD, but is also important for the induction of LTD-,IEi.e., (Figure 2D, E).[50]

Variants of PKC can be calcium-dependent or noncalcium dependent (For review see ref. [59]). Calcium dependent PKCs can be

activated by both calcium as well as DAG. Noncalcium dependent PKCs are only activated by DAG. A calcium dependent variant of PKC, PRKCA, is enriched in *Plcb4*+ PCs, whereas a noncalcium dependent PKC, PRKCD, is enriched in *Aldoc*+ PCs (Figures 3D, E and 4E, F).[44] While the implications of this have not been experimentally verified, it may suggest that, in *Plcb4*+ PCs, the induction of LTD is more sensitive to calcium, achieved by strong PF and CF activity. In contrast, in *Aldoc*+ PCs, LTD may be less dependent on calcium, but much more on the production of DAG, through pathways such as mGluR1-PLCB3 activation.

Possibly, the increased expression of PRKCA may lower the overall threshold for LTD induction in *Plcb4*+ PCs. This may point to potential differences in presynaptic activity required for the induction of LTD. *Aldoc*+ PCs need to optimize signals for the production of DAG, whereas *Plcb4*+ PCs require signals optimal for the production of DAG and/or the influx of calcium.

The activation of PKC is also regulated by the balance of DAG and its metabolite, phosphatidic acid (PA). DAG produced by the activation of mGluR1-PLCB is regulated by enzymes called diacylglycerol kinases (DGKs). DGKs regulate the concentration of DAG and phosphatidic acid (PA), through conversion of DAG into PA (Figures 3D, E and 4E, F).[112] This gives these enzymes an important role in regulating plasticity.[112,113] In PCs, DGKZ is essential for the induction of PF-PC LTD, as they regulate the activity of PKCa (*Prkca*).[114] Notably, DGKB, DGKI, and DGKH are co-expressed with *Plcb4*, whereas *DGKH* is co-expressed with *Aldoc* (Figures 3D, E and 4E, F).[44] Specialized regulation of DAG/PA balance, and its effects on downstream activators such as PKC, may thus play a role in the balancing between LTD/LTP.

Role of nitrous oxide signaling in plasticity in different Purkinje cell populations

Nitrous oxide signaling plays an important role in the induction of both LTP and LTD (Figure 2).[21] NO is synthesized presynaptically, but this synthesis is triggered by activity of presynaptic endocannabinoid (ECB) receptors. ECB receptors are activated by 2-AG, which is produced and diffuses from the postsynaptic side. Presynaptically produced NO diffuses to the postsynaptic membrane, where it promotes the production of AA by PLA2, or activates N-ethylmaleimide-sensitive factor (NSF).[62] NSF promotes AMPA receptor insertion, whereas AA promotes PKC activity and thus AMPA receptor endocytosis (Figure 2D, E).[54] Thus, during LTD, NO signaling supports PKC activity through increased AA production. During LTP, continuous PF activity facilitates stable activity of NSF.

No specific molecules directly involved in NO signaling (i.e., PLA2, NSF) are enriched in *Aldoc*+ or *Plcb4*+ PCs. However, NO signaling is reliant on calcium. PLA2, which produces AA, which is then further converted into DAG, is activated by calcium. Thus, NO signaling may also be different, primarily due to differences in calcium dynamics (Figures 3D, E and 4E, F).

Neuronal excitability is regulated by specific ion channels

Intrinsic activity in PCs is generated mainly by voltage gated sodium (I_{Na}), voltage gated calcium (I_{Ca}), voltage gated potassium (I_{Kv}), and calcium activated potassium (I_{KCa}) currents (Figure 4D).[115,116] Intrinsic activity is maintained by a resurgent I_{Na} current, which reopens due to repolarization of the membrane.[116,117] Repolarization is mainly facilitated by I_{Kv} and I_{KCa}, specifically large (BK) and small (SK) conductance calcium activated potassium channels.[116] Ca^{2+} influx is facilitated by I_{Ca}, primarily through P/Q-type channels.[118,119] SK channels are especially important for the excitability of PCs, as they control the after hyperpolarization (AHP) observed in action potential bursts.[105,119]

Many ion channels or ion channel subunits are enriched in either *Plcb4*+ or *Aldoc*+ PCs (Figure 4D). Notable examples include the voltage-gated sodium channel *Scn9a* (Na_v 1.7), which is enriched in *Plcb4*+ PCs.[44] Increased sodium conductance through voltage-gated sodium channels may contribute to the increased SS rate of *Plcb4*+ PCs. *Plcb4*+ PCs also express fewer SK2 channels, and have a relatively small AHP.[31,44] Thus, differences in SK2 channel expression may contribute to the differences in simple spike frequency between *Plcb4*+ and *Aldoc*+ PCs. *Cacna1d*, whose expression is enhanced in *Aldoc*+ PCs, is known to negatively regulate neuronal excitability in the amygdala.[98] Thus, increased expression of the VGCCs *Cacna1c* and Cacna1d in *Aldoc*+ PCs may reduce overall intrinsic activity through increased activation of SK2. In *Plcb4*+ PCs, fewer VGCCs activate fewer SK2 channels, contributing to a higher intrinsic simple spike frequency. A variety of voltage-gated potassium channel subunits are also enriched in either *Plcb4*+ or *Aldoc*+ PCs (Figure 4D).[44] The majority associate with the *Plcb4*+ population, indicating that perhaps faster or more regulated repolarization also contributes to the increased intrinsic activity of *Plcb4*+ PCs.

Plasticity of intrinsic activity differs between Purkinje cell populations

Aside from the synaptic plasticity discussed so far, the intrinsic excitability of PCs may also undergo a form of plasticity, termed long term potentiation- or depression of intrinsic excitability (LTP-IE, LTD-IE) (Figure 4A, B) ([27,120], for review see ref. [51]). LTP-IE is driven by continuous high activity, either from synaptic activity of PFs, or by high-frequency stimulation of PCs,[31,120] whereas LTD-IE is induced by paired stimulation of PF and CF.[27] Note that these induction criteria are essentially identical to those of required for PF-PC LTP and LTD. Therefore, PF-PC LTP and LTP-IE on the one hand, and LTD and LTD-IE on the other, occur simultaneously.[27,120]

The molecular mechanisms that drive LTP-IE and LTD-IE are also remarkably similar to those of PF-PC LTP and LTD (Figure 2A, B), respectively. Plasticity of intrinsic excitability relies on the modulation of SK2 channels, altering the excitability of specific dendritic branches.[121] During LTP-IE the removal of SK2 channels

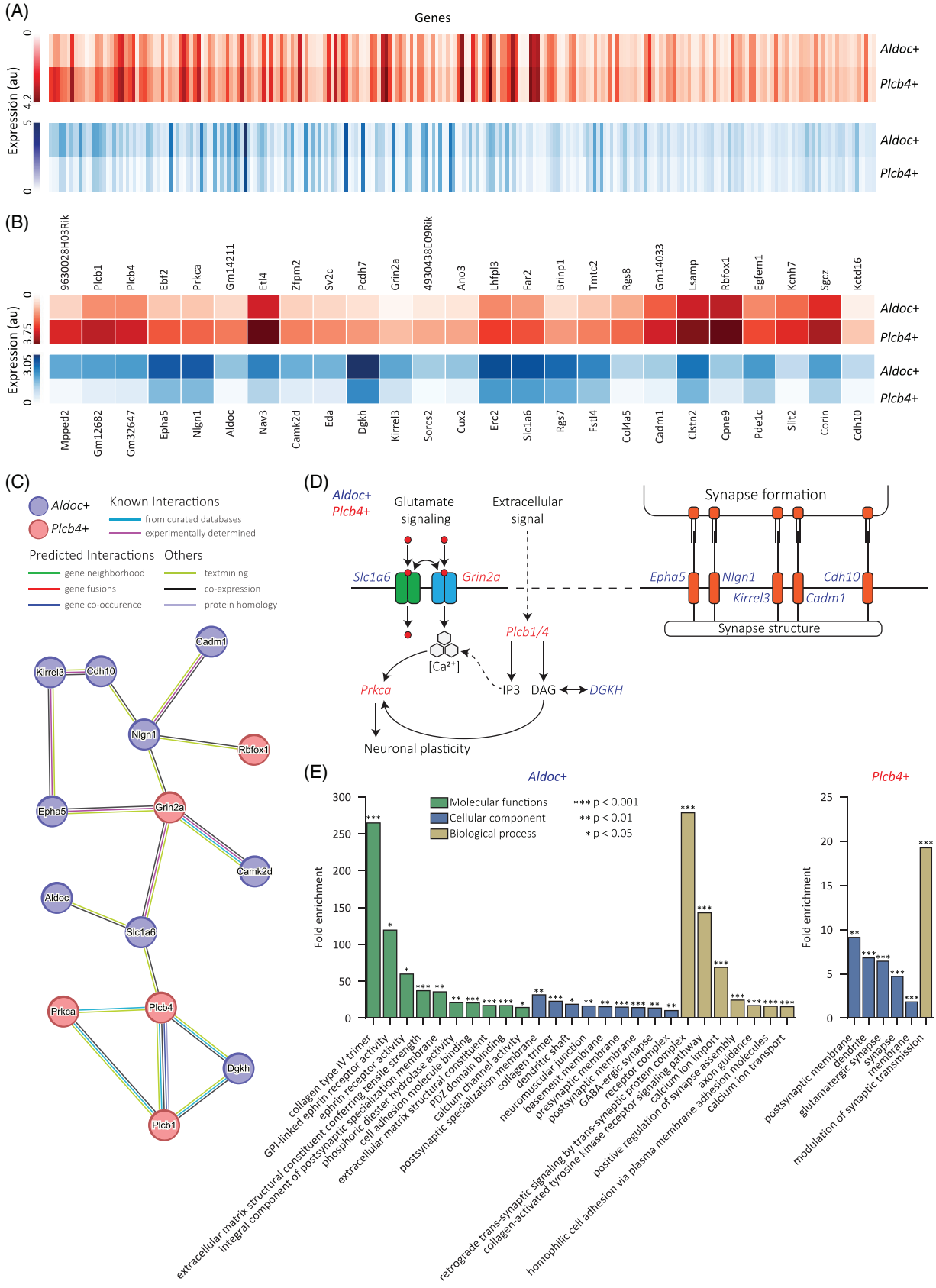

FIGURE 5 Analysis of the most differentially expressed genes co-expressed with *Aldoc* and *Plcb4*. (A) Gene expression in two PC subtypes associated with either *Plcb4* or *Aldoc*, as reported by ref. [44]. A subset of genes is enriched in *Plcb4*+ PCs (b, top, red), and another in *Aldoc*+ PCs (b, bottom, blue). (B) Gene expression in *Aldoc*+ and *Plcb4*+ PCs of the top 25 most differentially expressed genes with *Aldoc* (25) and *Plcb4* (25). All genetic data were adapted from ref. [44]. (C) STRING network of the top 25 most differentially expressed genes (25 *Aldoc*/25 *Plcb4*). Genes

increases intrinsic excitability by reducing the AHP.[31,105,122] LTP-IE relies on calcium influx, and the activation of PP1, PP2A, and PP2B (Figure 3F).[51,120,123] The exact modification that occurs during LTD-IE is not clear, although the modification of BK channels has been suggested as a potential mechanism (Figure 4E).[51] LTD-IE requires intracellular calcium signals and PKC activation, but is independent of SK2.[27] CaMKII may also play an important role in LTD-IE by modifying BK channels.[51] While specific cerebellar zones show increased or decreased propensities for the induction of LTP-IE this is not known for LTD-IE[31] *Plcb4*+ zones have an increased propensity for LTP-IE although it is currently unclear whether this also extends to LTD-IE. Many molecules of the previously described signaling pathways are co-expressed with either *Aldoc* or *Plcb4* (PF-PC plasticity; Figure 3D, E – Intrinsic plasticity; Figure 4D–F).[42,44]

Pathway and protein interaction analyses reveal networks of differentially expressed genes in Purkinje cell populations

Despite an accelerated increase in our understanding of the molecular make-up of PCs, the association between molecular expression and their physiological and plasticity properties remains mostly unclear. Advances in single cell RNA sequencing techniques have indeed revealed hundreds of differentially expressed genes in PCs.[42–44] To investigate the association between molecular expression and physiological properties of PC, we performed an analysis of gene expression profiles in different populations of PCs. For this analysis we used molecular expression data reported by recent RNA sequencing studies.[42–44]

Based on RNA sequencing studies, 229 individual genes are co-expressed with *Plcb4*, and 246 with *Aldoc* in PCs (Data S1, Figure 5A).[44] Out of all differentially expressed genes, we selected the top 25, 50, and 75 genes whose expression differed most between *Aldoc*+ and *Plcb4*+ PCs (Figure 5B, Figure S1). First, we generated protein interaction networks using the STRING database[124] (https://string-db.org/, Figure 5C, Figure S1). Out of the 50 (25 *Plcb4*/25 *Aldoc*) genes (Figure 1E), a coherent network with 16 heavily modulated genes and prominent interactions formed (Figure 5C). Most of these genes have clear physiological functions within PCs (Figure 5D). *Kirrel3*, *Nlgn1*, *Cadm1*, *Cdh10*, and *Epha5* are involved in the formation, maintenance, and restructuring of cell-cell contacts and synapses.[125–129] *Plcb1* and *Plcb4* catalyze a chemical reaction which forms inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4-5-biphosphate (PIP2), which provide essential signals for the induction of synaptic plasticity, such as the activation of *Prkca*.[50,53,56] The concentration of DAG is further regulated by diacylglycerol kinase (*DGKH*).[112,113] Other genes are also involved in the signaling cascade of synaptic plasticity, such as the NMDA receptor subunit *Grin2a*, which facilitates calcium influx, the glutamate transporter *Slc1a6* which limits the activation of other glutamate receptors, and *IGF1*, which supports the activity of *Prkca* through ERK signaling.[29,50,74,130]

In addition, we analyzed how the expression of these genes changes over the course of development (Figure 6). A human dataset [NCBI GSE22569[131]] with gene expression data from the cerebellum aged between 2 days and 90+ years shows distinct regulation of gene expression. Although this dataset is not PC specific, and contains only human data, it illustrates the regulation of distinct genes during critical developmental stages, highlighting how learning related activity involving *Aldoc* and *Plcb4* becomes active after approximately 196–443 days. Many genes such as *Aldoc* and *Grin2a* are initially downregulated, but between 19 and 94 days after birth, they are upregulated. In contrast, genes such as *Prkca* and *Kirrel3* are initially upregulated, but are downregulated between 19 and 196 days. Up- and downregulation of differentially expressed genes over the course of development, especially early in life, may thus play an important role in shaping and maintaining learned behaviors.

An important transcription factor associated with the expression *of Aldolase C* is *ZBTB20*. Zinc Finger and BTB Domain Containing proteins (ZBTBs) are part of a family of proteins consisting of many transcriptional regulators. Indeed, *ZBTB20* also plays a role in controlling the RNA expression of genes. This homeobox gene is known to play a role during development in neuronal differentiation, maturation but also circadian biology.[132] Therefore, it orchestrates RNA expression of many transcripts within the two pools of cells under investigation; *Aldoc*+ versus *Plcb4*+. *ZBTB20* has been linked to cognitive function, since *ZBTB20* expression can impact learning and memory. Last but not least *ZBTB20* plays a role in regulating energy metabolism, and alterations in its expression have been associated with metabolic disorders. *ZBTB20* regulates plasma triglyceride metabolism by repressing lipoprotein lipase gene transcription.[133] The connection between metabolism and circadian rhythm is bidirectional and multifaceted, with each influencing the other to maintain metabolic homeostasis and optimal health. Disruptions to circadian rhythms, such as those caused by shift work, jet lag, or irregular sleep patterns, can lead to metabolic dysregulation and increase the risk of metabolic disorders.

co-expressed with *Aldoc* are colored blue, and genes co-expressed with *Plcb4* are colored red. The top 25 most differentially expressed genes were entered into the STRING database (https://string-db.org/). The minimum required interaction score was 0.400. Only genes with interactions are shown. For more detail on the STRING database see ref. [124]. (D) Simplified scheme of the molecular functions of most genes shown in panel A. Genes with the highest modulation in *Aldoc*+ PCs are involved in synaptic structure, synapse formation, and cell-cell contacts. Genes with the highest modulation in *Plcb4*+ PCs are mostly involved in neuronal plasticity. (E) GO enrichment (https://geneontology.org/) analysis of the 50 most differentially expressed genes (50 *Aldoc*/50 *Plcb4*). Genes co-expressed with *Aldoc* are associated with cell-cell interaction, synapse formation, and synaptic structure, whereas genes co-expressed with *Plcb4* are associated with synaptic functions and long term plasticity. Enriched GO terms for GO molecular function, cellular component and biological process with FDR < 0.05 (PLCB4) or FDR < 0.05 and fold enrichment > 10 (ALDOC) are shown.

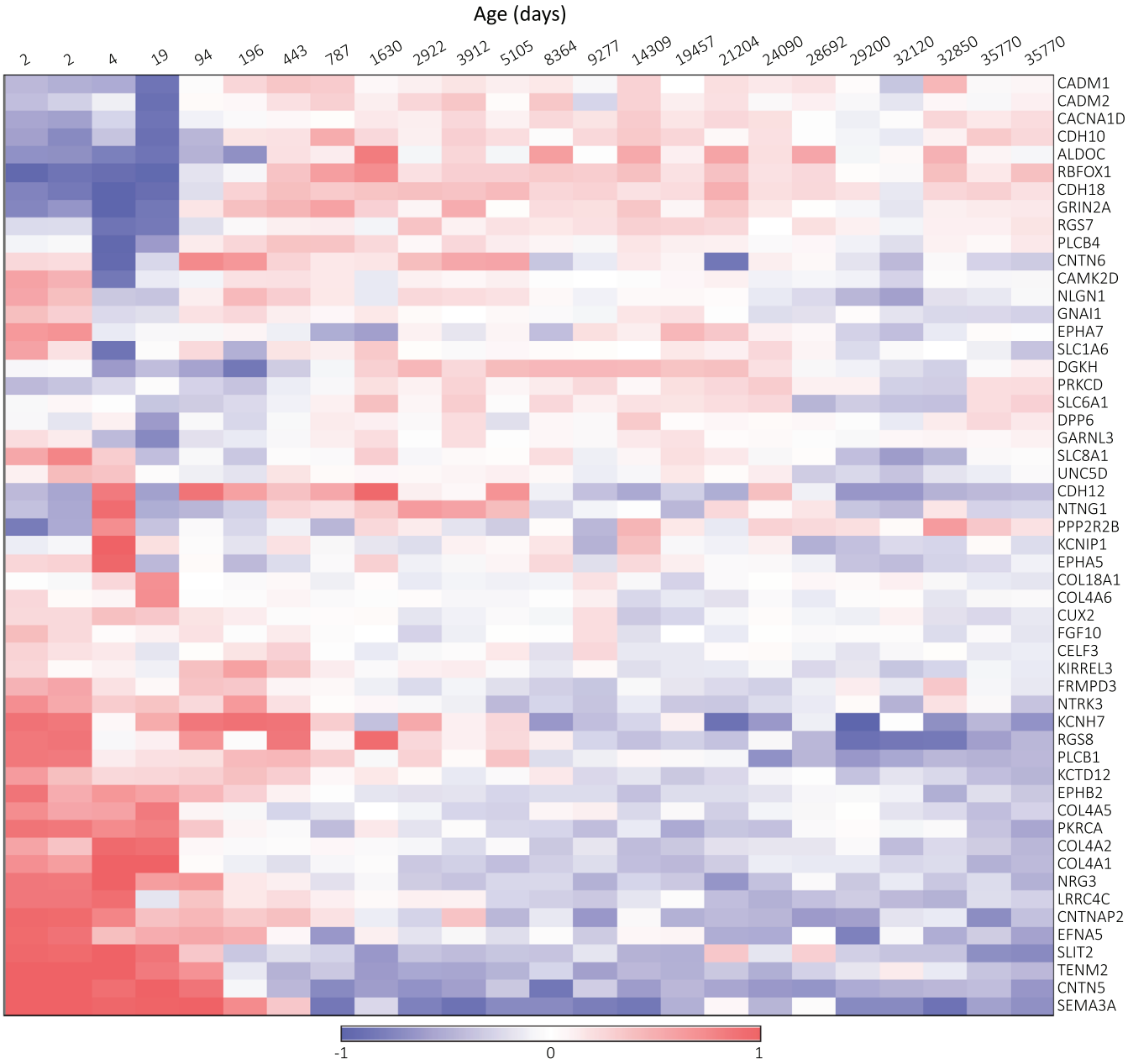

FIGURE 6 Heatmap of the most differentially expressed genes co-expressed with *Aldoc* and *Plcb4* during human cerebellar development. OmniViz Treescape showing the expression in human postnatal cerebellar cortex throughout lifespan. Data are from human postmortem brain samples from the cerebellar cortex [NCBI GSE22569[131]]. Gene expression levels are shown in a heatmap where red means relative upregulation compared to the Geometric mean. In blue are shown the down-regulated genes compared to the geometric mean. The color intensity correlates with the degree of change. Human homologs of the mouse transcripts were downloaded from NCBI Homologene and shown in the total RNA cerebellum dataset. Genes included are the top 50 most differentially expressed genes co-expressed with *Aldoc* or *Plcb4*[44] (Same as in Figure 5b). Some genes are initially downregulated, but are upregulated between day 19 and 94, whereas other genes are initially upregulated, but are downregulated between day 19 and 196.

Further analysis of the most extensively modulated genes was aimed at determining the major biological themes associated with molecular expression in either *Aldoc*+ or *Plcb4*+ PCs (Figure 2C). For this, we selected the 50 genes whose expression correlated best with either *Aldolase C* or *Plcb4*. The molecular functions (MF), biological pathways (BP), and cellular components (CC) of genes within *Aldoc*+ PCs are dominated by two themes, the regulation of cell-cell interactions, and the regulation of calcium (Figure 2C, left). In contrast, *Plcb4*+ genes associate with specialization of the synapse, and synaptic plasticity (Figure 2C, right). The genetic specialization of PCs into two distinct subpopulations thus plays an important role in determining the physiological properties of a PC. In particular, the *Plcb4*+ PC populations propensity for plasticity is evident from both our genetic analysis and previous physiological data. In the network generated from the top

25 most regulated genes, 7 have roles in the induction of synaptic plasticity in PCs (Figure 2B). *IGF1*, *Plcb1/4*, *Prkca*, and *Grin2a* are all co-expressed with *Plcb4*, and positively regulate the induction of plasticity. These results illustrate that the genetic specialization of PCs has clear functional consequences, and also support our previous analysis of specific molecular mechanisms that regulate, for example, LTD. Most clear is that there is some specialization of the synapse or neural membrane, as shown by our bioinformatics analyses, and from the descriptions of specific molecular mechanisms.

CONCLUSION AND PROSPECTS

The highly specialized molecular make-up of PCs clearly plays an important role in determining the physiological properties of a PC. We have highlighted that the expression profile of individual PCs shapes their activity, as well as their propensity for plasticity. There are several molecular differences which drive these physiological differences observed in *Aldoc*+ versus *Plcb4*+ PCs. In particular, there are differences in the molecular pathways regulating calcium dynamics, which are critical for the induction of PF-PC plasticity and intrinsic plasticity, and a key regulator of intrinsic excitability. These molecules include *mGluR1b*, *EAAT4*, *Gaq*, *Plcb4*, *Plcb3*, *Plcb1*, *ITP3R*, *RyR1/2*, *Cacna1c*, *Cacna1d*, *GRIN2A*, *TRPC3*, *GRID2*, *GluA2 and STIM1*.

The specialization of molecular expression clearly has a physiological function, although it is not yet clear why molecular specialization is important for overall cerebellar function. Clearly, both *Aldoc*+ and *Plcb4*+ PCs are capable of effectively modulating their simple spike rate. However, the rate at which this modulation occurs, as well as its likelihood and direction, likely follow distinct patterns defined by the individual PCs molecular identity, as well as their up- and downstream circuitry. In the future, it will be important to further investigate how molecular expression changes over the course of development, and how these changes affect plasticity and intrinsic activity. Furthermore, the classification of more subtypes of PCs will likely reveal subtypes with different physiological properties from those already identified. Future work will have to decipher the roles of individual genetic components highlighted in this article. Further elucidation of the transcription regulation in PC subpopulations of cells may also provide additional insight in pathway changes over time that can affect learning and memory in general.

LIMITATIONS

Our review aims to provide a focused discussion on the effects of transcriptome on neuronal function. However, there are a few key limitations we wish to discuss. First, enhanced expression of specific RNA transcripts does not necessarily equate to enhanced protein translation. Thus, the direct link between the transcriptome of a neuron and its physiology is limited. While we make the assumption that the expression of RNA transcripts leads to translation and therefore protein expression, future confirmation (using, e.g., immunohistochemistry) of specific proteins will have to provide conclusive evidence on the relative expression of specific proteins in *Aldoc*+ and *Plcb4*+ PCs. In addition, the effects of epigenetic modification have not been considered in our analyses. The epigenetic modification of DNA alters the structure of chromosomes, affecting the transcription of DNA. Furthermore, it is known that epigenetics can affect synaptic plasticity.[134] Epigenetic modification therefore likely plays an important role in the development and maintenance of differential gene expression, as well as the physiological phenotype of Purkinje cells. Specific genes which regulate epigenetic modification may be specifically associated with the expression of *Aldoc*, such as the histone deacetylases *HDAC9* and *HDAC7*.[44] The inhibition of *HDAC* has been associated with the enhancement of LTP in the hippocampus.[134,135] Future investigation of epigenetic modification may provide further insight into the development and functional properties of *Aldoc*+ and *Plcb4*+ PCs.

Second, There are also caveats related to the interpretation of experimental outcomes of physiological studies, as different experimental conditions may influence the outcomes. For example, in vitro patch-clamp recordings performed at different environmental temperatures (e.g., physiological vs. room temperature) with different concentrations of ions, and with different animal species, strains, and ages can heavily influence the experimental outcomes.

Last, in this review we discuss PCs as belonging to one of two subpopulations, namely, *Aldoc*+ or *Plcb4*+. However, it is known that additional "sub" profiles of PCs with complex molecular expression patterns further divide PCs. For example, PCs in the flocculus region of the cerebellum lack clearly defined boundaries of *Aldoc* expression, but are subdivided based on the expression of KCTD12.[109] Physiologically, KCTD12+ and *Aldoc*+ PCs also differ from KCTD12- and *Aldoc*+ PCs.[109] Other research has identified nine clusters of PCs, of which two associate with the expression of *Aldoc* while seven associate with the expression of *Plcb4*.[43] It is therefore an oversimplification to assume that PCs are divided into only two categories. Despite this, the division of PCs into either *Aldoc* or *Plcb4* expressing PCs has clear physiological and functional implications, and plays an important role in cerebellar functioning.

AUTHOR CONTRIBUTIONS

Writing of the manuscript: Stijn Voerman, Robin Broersen, Sigrid M. A. Swagemakers, Peter J. van der Spek, Chris I. De Zeeuw. Design and conceptualization: Peter J. van der Spek, Stijn Voerman, Robin Broersen, Chris I. De Zeeuw, Sigrid M. A. Swagemakers. Bioinformatics: PJVDS, Sigrid M. A. Swagemakers. Figure illustration and design: Stijn Voerman, Peter J. van der Spek, Sigrid M. A. Swagemakers, Robin Broersen, Chris I. De Zeeuw.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All figures were generated using custom Python code (Python 3.8.18). All data and code used for the generation of the figures is freely available at https://github.com/s-voerman/DEG-Project. For questions regarding data or code please contact s.voerman@erasmusmc.nl.

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