The secreted protein C1QL1 and its receptor BAI3 control the synaptic connectivity of excitatory inputs converging on cerebellar Purkinje cells

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Running Title:C1QL1/BAI3 and neuronal connectivity

Summary

Precise patterns of connectivity are established by different types of afferents on a given target neuron, leading to well-defined and non-overlapping synaptic territories. What regulates the specific characteristicsof each type of synapse, in terms of number, morphology and subcellular localization, remains to be understood. Here we show that the signalingpathway formed by the secreted complement C1Q-relatedprotein C1QL1 and its receptor,the adhesion-GPCR Brain Angiogenesis Inhibitor 3(BAI3),controlthe stereotyped pattern of connectivity established by excitatory afferentson cerebellar Purkinje cells. The

BAI3 receptor modulates synaptogenesis of both parallel fiber and climbing fiber afferents. The restricted and timely expression of its ligand C1QL1 in inferior olivary neurons ensures the establishment of the proper synaptic territory for climbing fibers. Given the broad expression of C1QL and BAI proteins in the developing mouse brain, our study revealsa general mechanism contributing to the formation of a functional brain.

Introduction

In the nervous system, eachtype of neuron is connected toits afferents in a stereotyped pattern that is essential for the proper integration of information and brain function. A neuron can receive several convergent inputs from different neuronal populations with specific characteristics. The number and the subcellular localization of synapses from each afferent on a target neuron aredetermined by a complex developmental process that involves recognition, repulsion, elimination of supernumerary synapsesand/or guidance posts(Sanes and Yamagata, 2009; Shen and Scheiffele, 2010). How these precise patterns of connectivity are established is likely to varydepending on the neuronal population and remains a poorly understood question.

Several classes of adhesion proteins, such as cadherins, immunoglobulin-superfamily (IgSF) proteins,neuroligins and Leucine-Rich Repeats transmembrane (LRRTM) proteins, have been involved in synapse formation, maturation and function(Shen and Scheiffele, 2010). In addition, secreted proteins, such as WNTs(Salinas, 2012), pentraxins (Sanes and Yamagata, 2009; Shen and Scheiffele, 2010; Sia et al., 2007) or CBLNs (Yuzaki, 2011a), can regulate synapse formation and function, both in an anterograde and retrograde manner. This molecular diversity and functional redundancy is in agreement with the idea that a specific set of molecular pathways defines each combination of afferent-target neuronin the vertebrate brain(O'Rourke et al., 2012; Sperry, 1963).

Molecular signaling pathways regulate different aspects of synapse specificity. Adhesion proteins, such as IgSF members Sidekicks in the retina(Yamagata and Sanes, 2008), can have an instructive role for the choice of the synaptic partners, and also determine the balance of inhibitory versus excitatory connectivity, as illustrated by the studies ofneuroligins(Südhof, 2008).Further specificity resides in the definition of non-overlapping territoriesfor inhibitory

and excitatory synapses on a given neuron. For example, Purkinje cells receive two types of excitatory inputs (parallel fibers from granule cells and climbing fibers from inferior olivary neurons) and two types of inhibitory inputs (from basket cells and stellate cells), which form synapses on separate and non-overlapping territories. Adhesion proteins from the L1 Ig subfamily have been shown to control the specific subcellular localization of each inhibitory synapse (Ango et al., 2004; 2008).A very recent studyof Ce-Punctin, an ADAMTS-like secreted protein, in the invertebrate nervous system has shown that specific isoforms are secreted by cholinergic and inhibitory inputs and control the proper localization of corresponding synapses at the neuromuscular junction(Pinan-Lucarré et al., 2014). Thus, in addition to adhesion proteins, the specific secretion of some factors could play an important role in defining synapse specificity.

In the vertebrate brain, the complement C1Q-relatedproteins compriseseveral subfamilies:proteins related to the innate immunity factor C1Q,some of which have been involved in synapse elimination(Stevens et al., 2007), CBLNs known for promoting synapse formation (Yuzaki, 2011a)and the C1Q-like (C1QL) subfamily. Proteins of this last subclass were recently shown to be high-affinity binding partners of the adhesion-G protein-coupled receptor (GPCR) Brain Angiogenesis Inhibitor 3 (BAI3), and to promote synapse elimination in cultured hippocampal neurons(Bolliger et al., 2011). Our understanding of the function of Brain Angiogenesis Inhibitor receptors in synaptogenesis is limited. The BAI3 receptor has been identified in biochemical preparations of synapses both in the forebrain (Collins et al., 2006) and in the cerebellum(Selimi et al., 2009), and recently BAI1 was shown to promote spinogenesis and synaptogenesis through its activation of RAC1 in cultured hippocampal neurons(Duman et al., 2013). Interestingly, the BAI proteins have been associated with several psychiatric symptoms by human genetic(DeRosse et al., 2008; Liao et al., 2012)or functional studies(Okajima et al., 2011) and could thus directly be involved in the synaptic

defects found in these disorders. In the present study, we explored the role of the C1QL/BAI3 signaling pathway in the establishment of specific neuronal networks, using a combination of expression and functional studies in the developing mouse brain. Our results show that the temporally and spatially controlled expression of C1QL1 and the presence of its receptor, the adhesion-GPCRBAI3, in target neurons arekey determinants ofexcitatory synaptogenesis and innervation territories in the vertebrate brain.

Results

The spatiotemporal expression pattern of the C1QL ligands and their BAI3 receptoris in agreement with arole inneuronal circuit formation

The adhesion-GPCR BAI3 has been found at excitatory synapses by biochemical purifications(Collins et al., 2006; Selimi et al., 2009). In transfected hippocampal neurons, BAI3 is highly enriched in spines and is found to colocalize with and surround clusters of the postsynaptic marker PSD95 using immunocytochemistry (figure S1).Together with the fact that BAI receptors can modulate RAC1 activity, a major regulator of the actin cytoskeleton,in neurons(Duman et al., 2013; Lanoue et al., 2013), these datasuggest a functionfor the BAI3 receptor in the control of synaptogenesis. To play this role, the timing and pattern of BAI3 expression should be in agreement with the timing of synaptogenesis. *In situ* hybridization experiments showed that*Bai3*mRNAs are highly expressed in the mouse brain during the first two postnatal weeks, in regions of intense synaptogenesis such as the hippocampus, cortex and cerebellum (figure 1A). In the cerebral cortex, a gradient of *Bai3* expression is observed with the highest level at postnatal day 0 (P0) in the deep layers and at P7 in the most superficial layer, reminiscent of the inside-out development of this structure.At these stages *Bai3* is also expressed in the brainstem, in particular in the basilar pontine nucleus and the inferior olive, and in the cerebellum (figure 1A). In the adult mouse brain, *Bai3* expression

decreases in many regions, such as in the brainstem (assessed by quantitative RT-PCR, figure 1B), and becomes restricted to a few neuronal populations, such as cerebellar Purkinje cells, pyramidal cells in the hippocampus and neurons in the cerebral cortex (figure 1A and figure S2).

Secreted C1QL proteins of the C1Q complement family can bind the BAI3 receptor with high affinity(Bolliger et al., 2011), and could thus regulate its synaptic function. *In situ* hybridization experiments(figure 1),in accordance with previously published data (Iijima et al., 2010), show that *C1ql*mRNAs, in particular *C1ql1* and *C1ql3*,are highly expressed during the first two postnatal weeks in variousneuronal populations. *C1ql3*mRNA is found in the cortex, lateral amygdala, dentate gyrus and deep cerebellar nuclei.*C1ql1* is very highly expressed in the inferior olive at all stages,includingin the adult.Itis also foundat P0 and P7 in neurons of the hippocampus, cerebral cortex and in few other neurons of the brainstem. By quantitative RT-PCR, we also detected *C1ql1*expression in the cerebellum, with a peak at P7 at a level that is 5 fold less than in the brainstem. This transient cerebellar expression is in agreement with previous *in situ* hybridization data that showed expression of *C1ql1* in the external granular layer of the developing cerebellum(Iijima et al., 2010).

This expression analysis shows that C1QL proteins are produced inneuronsthat are well-described afferents of neurons expressingBAI3, such asinferior olivary neurons that connect Purkinje cells (PCs). It also indicates thatdifferent C1QL/BAI3 complexes could control synaptogenesis in various regions of the brain. The C1QL3/BAI3 complexis prominent in the cortex and hippocampus, whilethe C1QL1/BAI3complex might be particularly important for excitatory synaptogenesis on cerebellar PCs. Indeed the expression pattern of the C1QL1/BAI3 couple correlates with the developmental time-course of excitatory synaptogenesis in Purkinje cells: these neurons receive their first functional synapses from the

climbing fibers, the axons of the inferior olivary neurons, on their somata around P3, at a time when *C1ql1* mRNA expression starts to increase sharply (figure 1A and 1B) and when *Bai3* mRNA is already found in PCs (figures1 and S2). PCs are subject to an intense period of synaptogenesiswith their second excitatory inputs, the parallel fibers, starting at P14, when*Bai3* expression in the cerebellum reaches its maximum (figure 1B). Given the well-described timing and specificity of PC excitatory connectivity, we focused our studies onthe olivocerebellar network to identify the function of the C1QL/BAI3 complexes during the formation of neuronal circuits.

The adhesion-GPCR BAI3 promotes the development of excitatory synaptic connectivityon cerebellar Purkinje cells

Inferior olivary neurons (IONs) send their axons to the cerebellum where they start forming functional synapses on somata of PCs ataround P3. These projections mature into climbing fibers (CFs) while PCs develop their dendritic arborduring the second postnatal week.Starting at P9, a single CF translocates and forms a few hundred synapses on thorny spines of PC proximal dendrites(Hashimoto et al., 2009).Each PCalso receives information from up to 175.000 parallel fibers (PFs) through synapses formed on distal dendriticspines,in particularduring the second and third postnatal weeks(Sotelo, 1990).To testthe role of the BAI3 receptor during the development of the olivocerebellar network, we developedan RNA interference approach:two different short hairpin RNAs targeting different regions of the *Bai3* mRNA (shBAI3)weredesigned and selected after testing theirefficiency in transfected HEK293 cells (data not shown). A lentiviral vector was then used to drive their expression in neurons both *in vivo* and *in vitro*, together with the green fluorescent protein (GFP, under the control of the ubiquitousPGK1 promoter). In mixed cerebellar culturestransduced at 4days*in vitro*(DIV4),both shRNAs led to about 50% knockdown of *Bai3*by DIV7, and did notaffect

the expression level of another PC-expressed gene, *Pcp2*, confirming their specificity(figure S3A). Knockdown of *Bai3* wasstill presentafter 10 days in culture(figure S3A). Morphological analysis in mixed cerebellar cultures confirmed that both shRNAs against *Bai3*induced the same phenotype (cf. below). Since one of the shRNA constructs was more efficient(similar levels of knockdown with half the amount of lentiviral particles), it was chosen for *in vivo* experiments.

Recombinant lentiviral particles driving either shBAI3 or a control non-targeting shRNA (shCTL) wereinjected in the molecular layer of the cerebellum ofmouse pups at P7, when the most intense period of PF synaptogenesis starts and just before the translocation of the strongest CF(Hashimoto et al., 2009). *Bai3* knockdown induced visible deficits inthe connectivity between CFs and their target PCsvisualized at P21 using an antibody against vGluT2, a specific marker of CF presynaptic boutons in the molecular layer(figure 2). The extension of the CFsynaptic territory on arbors of PCs expressing shBAI3 was reduced by about 35% when compared to shCTL-expressing PCs (figure 2A and 2C).This effect is cell-autonomous since it is not observed in non-GFP PCs in the transduced region (Figure S4A). Quantification of synaptic puncta revealed a reductionin number (507.75±109.94 vs 217.10±37.21; * $p \leq 0.05$, Student unpaired *t* test) and volume (about 30%) of vGluT2 clusters on shBAI3-PCs when compared to shCTL-PCs (figure 2).These morphological changes were accompanied by a deficiency in CF transmission, as shown by the reduced whole cell currents elicited byCF stimulation in PCs recorded in acute cerebellar slices from P18 to P23 mice (figure 2D, shCTL=-2122.54 pA ± 204.77, n = 5 cells; shBAI3=-1478.6 pA ± 186.24, n = 8 cells; Student *t* test, *$p < 0.05$).

A reduced spine density was also evident at P21 in distal dendritesof shBAI3-PCs(figure 3A), suggesting a potential defect in parallel fiber (PF) connectivity. To confirm this, we recorded

PF-EPSCs of PCs and input-output relationships were examined. Their amplitudes gradually increased with PF stimulus intensity but reached a plateau for much smaller values of stimulation in BAI3-deficient PCs than in control PCs (figure 3B). The high density of PF synapses in the cerebellar molecular layer impedes precise morphological quantifications of synaptic defects in transduced PCs*in vivo*. We thus turned to mixed cerebellar cultures that recapitulate PF synaptogenesis with similar characteristics as *in vivo* since, in this system,PCs develop highly branched dendrites studded with numerous spines on which granule cells form synapses. The effect of *Bai3* knockdown on PF/PC spinogenesis and synaptogenesis was assessed atDIV14,10 days post-transduction,by co-immunolabeling followed by high resolution confocal imaging and quantitative analysis.An antibody against the soluble calcium binding protein CaBP allowed us to label PCs dendrites and spines, andan antibody against the vesicular transporter vGluT1, was used to label specifically the PF presynaptic boutons. A reduced spine density and a decreased mean spine head diameter was measured on 3D-reconstructed dendritesafter transduction ofPCswith either of the two shRNAs targeting*Bai3* (32%and 22%for shRNA#1 and shRNA#2 respectively, whencompared to shCTL, cf. figure 3D and figure S5). A significant reduction in the density ofPF contactswas also revealed in shBAI3-PCs compared to controls,at a level similar to the one observed for spine density(24%and 22%for shRNA#1 and shRNA#2 respectively, cf.figure 3E and figure S4C). Both shRNAs against *Bai3* induced similar defects. These reductions in spine and synapse density were not observed in non-transduced (non-GFP) PCs in transduced mixed cultures, showing that the effect of *Bai3* knockdown was cell-autonomous (figure S4B and S4C). Theseresults show that the adhesion-GPCR BAI3 regulates PF connectivity on PCs by controlling spinogenesis and synaptogenesis.

Thus the adhesion-GPCR BAI3 is a general promoter ofexcitatory synaptogenesis during development of the olivocerebellar circuit, since it controls the connectivity of both parallel fiber and climbing fiberexcitatory inputs on cerebellar PCs.

The ligand C1QL1 is indispensable forclimbing fiber/Purkinje cellsynaptogenesis

In the developing olivocerebellar circuit,*C1ql1*is expressed at high levels by IONs. The deficits in CF/PC synaptogenesis induced by knockdown of the adhesion-GPCR BAI3 suggested that the secretion of its ligand C1QL1 by climbing fibers could also regulate this process.An RNA interference approach was developed to target *C1ql1*by designing and selecting a shRNA efficient for *C1ql1* knockdown (shC1QL1) in transfected HEK293 cells (data not shown). To enable transduction ofneurons *in vitro* and *in vivo,* this shRNA was then integrated in a lentiviral vector co-expressing GPF under the ubiquitous PGK1 promoter.Quantitative RT-PCR analysis showed that a 90% reduction in *C1ql1* mRNA expression was induced by DIV7, 3 days post-transduction,an effect that was maintained at DIV14 (figure S3B).*C1ql1* expression levels could be entirely restored by co-transduction with lentiviral particles driving the expression of a resistant *C1ql1* cDNA construct under the PGK1 promoter, but not by a wild-type *C1ql1*construct (figure S3B).

The morphology and function of CF/PC synapses were assessed after injection of lentiviral particles driving shC1QL1 in the inferior olive of P4 neonates(figure S6).This stage corresponds to the beginning of CF synaptogenesis on PC somata and precedes their translocation on PC dendrites (figure S6).Compared to control shCTL-CFs that extended to 61% of the PC dendritic height by P14, there was a small but significant reduction in the extension of shC1QL1-CFs to about 56% (figure 4A and 4B). There was little difference in the proportion of translocating climbing fibers at P9 (11/35 for shCTL, 8/31 for shC1QL1, 14/46 for shC1QL1+Rescue; figure S6). These results suggest that *C1ql1* knockdown in IONs

has only a small effect on the ability of CFs to translocate. In contrast, the extension of the synaptic territory of shC1QL1-CFs, as assessed by anti-vGluT2 immunolabeling, was decreased by half compared to control shCTL-CFs (30%and 60% of PC dendritic height respectively, figure 4). The mean number of vGluT2 positive clusters per transduced CFwas also reduced by 50% by C1QL1 knockdown (figure 4). Co-transduction with lentiviral particles driving the expression of the resistant *C1ql1* constructcould partially rescue these phenotypes, showing that they were dependent on *C1ql1* expression (figure 4). To confirm these synaptic phenotypes at the electrophysiology level, CF-EPSCs were recorded in Purkinje cells in acute slices from animals injected with shC1QL1 and shCTL lentiviral particles. Recordings were performed in lobule II, a region targeted by transduced CFs.A49% decrease in CF transmissionwas observed inPCs from animals injected with shC1QL1 particleswhen compared to PCs from animals injected with shCTL particles (figure 4C; shCTL=-1771.27 pA ± 220.87, n = 8 cells, shC1QL1=-907.59 pA ± 131.67, n = 8 cells, Mann Whitney *U* test, *p < 0.05).Altogether these results show that*C1ql1* expression by CFsis indispensable for their normal connectivity on Purkinje cells.

Restriction of *C1ql1* expression to climbing fibers in the cerebellum is necessaryfor their proper innervation of the target Purkinje cell

The translocation of the “winner” CF on PC proximal dendrites starts at around P9 and continues until about P21 when the CFacquires its final synaptic territory (figure 2 and (Hashimoto et al., 2009)). At P7, just before CF translocation, the expression of*C1ql1* decreases in the cerebellum while it starts to increase in the brainstem to reach a plateau by P14 (figure 1). To assess whether thespecific expression pattern of *C1ql1* contributes to the acquisition of the final innervation territory of CFs on Purkinje cells, we misexpressed*C1ql1* in the cerebellum, by injecting lentiviral particles driving expression of a *C1ql1*cDNA (under

the control of the PGK1 promoter) in the molecular layerat P7(figure 5). The synaptic territory of CFs on PC dendrites was significantly reduced at P14 by *C1ql1* misexpression when compared to eGFP controls (vGluT2 puncta extending to 45% and 60% of PC height, respectively). Thus the restricted and specific expression of *C1ql1* by IONs that is progressively established during development is necessary for the development of the proper synaptic territory of the "winner" CF on the PC dendritic arbor.

The ligand C1QL1 promotes Purkinje cellspinogenesis in a BAI3-dependent manner

The deficits in PF spinogenesis and synaptogenesis induced by knockdown of the adhesion-GPCR BAI3 cannot be explained by its role in controlling CF/PC synaptogenesis. Since BAI3 has been identified at the PF/PC synapses(Selimi et al., 2009)and *C1ql1* is transiently expressed in the cerebellum(figure 1B and(Iijima et al., 2010)),the C1QL1/BAI3signaling pathway could directly regulate PC spinogenesis and PF synaptogenesis.We tested this hypothesis in cerebellar mixed cultures since the expression pattern of *C1ql1* in this systemis similar to the patternobserved *in vivo*, with a peak at DIV7(figure S7).As for its receptor BAI3, the effects of C1QL1 knockdown were assessed at DIV14,10 days post-transduction,using CaBP and vGluT1 immunostaining, high-resolution confocal imaging and quantitative analysis. Our results showa 47% reduction in PC spine density, a small but significant increase in spine head diameter,but no effect on the mean spine length, in shC1QL1 treated cultures compared to shCTLtreated ones (figure 6B). No change in the density of vGluT1 contacts on PC spines was detected,suggesting that the proportion of PFs able to synapse on the available spines remains stable, and that the reduction in spine density is overcome by an increase in the contact ratio between PFs and PCs in our culture system. All these effects were rescued by the concomitant expression of the resistant *C1ql1* cDNA construct but not bya wild-type *C1ql1*cDNA driven by the same PGK1 promoter (figure 6).

Thus C1QL1 secretion in the cerebellummodulatesspine production in PCs, thereby regulating the amount of postsynaptic sites available for innervation by parallel fibers.

C1QL proteins bind the BAI3 receptor with high-affinity(Bolliger et al., 2011), suggesting that C1QL1 could regulate spinogenesis in PCs through the adhesion-GPCR BAI3.In this case, the simultaneous knockdown of both proteins should not induce an additive phenotype. Knockdown of both *Bai3*and *C1ql1*, by co-transduction of cerebellar cultures with a mixture of lentiviral particles, led to a 30% reduction in spine density, similar to the one observed for knockdown of *Bai3* only (figure 7). Co-transduction of the control shCTL together with either shBAI3 or shC1QL1 induced the same level of spine reduction compared to shBAI3 or shC1QL1 alone(about 30% and 50%, respectively, figures 3, 6 and7), showing that there was no non-specific effect of co-transduction itself on spine density.A non-specific effect of co-expressing shC1QL1 and shCTL prevented the interpretation of the data on spine morphology (figure 7B). The level of reduction in spine density after double-knockdown correspond to the one detected for *Bai3* knockdown alone, which is smaller than for *C1ql1* knockdown alone. Thus, our results suggest that while C1QL1 and BAI3 do not control spine density independently, their regulation of this process is complex.

Discussion

Each neuron receives synapses from multiple types of afferents with specific morphological, quantitative and physiological characteristics. These patterns are stereotyped for each type of neuronal population and are key to the proper integration of signals during brain function. Here we show that the signaling pathway formed by the secreted protein C1QL1 and the adhesion-GPCR Brain Angiogenesis Inhibitor 3 regulates the development of proper excitatory connectivity on cerebellar Purkinje cells. First the BAI3 receptor promotes both parallel fiber and climbing fiber connectivity on Purkinje cells and is thus a general regulator of excitatory synaptogenesis. Second, the C1QL1 protein is indispensable for proper climbing fiber/Purkinje cell synaptogenesis and the development of the proper synaptic territory, but not for climbing fiber translocation.C1QL1also modulates the production of the final number of distal dendritic spines by Purkinje cells, therebyregulating the number of available contact sites for parallel fibers. Given the broad expression of the C1QL/BAI3 pathway in the developing brain, our study informsabout a general mechanism used for the control of brain connectivity.

Most excitatory synapses are made on dendritic spines. In the cerebellum, studies of mouse mutants such as *weaver* and *reeler*indicate that Purkinje cells can generate spines through an intrinsic program (Sotelo, 1990). While models involving the incoming axons in the process of spine induction have been put forward in other neuronal types such as cortical or hippocampal pyramidal cells, current data do not exclude an intrinsic program for spinogenesis in these neurons (Salinas, 2012; Yuste and Bonhoeffer, 2004). In all cases, the regulation of the actin cytoskeleton, in particular through modulation of RhoGTPases such as RAC1, is essential for the proper morphology and maturation of dendritic spines and associated synapses (Luo, 2002). The BAI receptors can regulate RAC1 activity both in

neurons (Duman et al., 2013; Lanoue et al., 2013) and other cell types (Park et al., 2007). Our results show that as shown for BAI1 in cultured hippocampal neurons (Duman et al., 2013), the adhesion-GPCR BAI3 regulates spinogenesis in distal dendrites of Purkinje cells *in vivo*. Purkinje cells produce two types of spines: a small number of thorny spines on the proximal dendrites that are contacted by climbing fibers, and very dense spines on the distal dendrites that are contacted by parallel fibers. In the adult cerebellum, PCsgenerate spines of the distal type in their proximal dendrites if the climbing fiber is removed through lesions or activity blockade(Rossi and Strata, 1995), showing an intrinsic ability to produce spines of this type. The adhesion-GPCR BAI3 could bepart of this intrinsic programsinceits expression is maintained at high levels in adult PCs, contrary to many other neurons. Transient expression of *C1ql1* in the external granular layer (figure 1 and(Iijima et al., 2010)), by a yet-to-be defined cell type,during Purkinje cell growth can modulate to a certain extent the number of spines produced in Purkinje cells, suggesting a local extrinsic regulation of the number of available contactsites for parallel fibers.

Various classes of membrane adhesion-proteins regulate the proper formation of mature excitatory synapses, including cadherins, neuroligins, and SynCAM (Shen and Scheiffele, 2010).Besides the well-described role of neurotrophins, increasing evidence also shows a role for other classes of secreted proteins, such as WNTs(Salinas, 2012) or complement C1Q-related proteins(Yuzaki, 2011a). The complement C1Q-related family is composed of three different subfamilies, the classical C1Q-related, the cerebellins (CBLN) and the little studied C1Q-like (C1QL) proteins. The classic C1Q complement protein promotes synapse elimination in the visual system (Stevens et al., 2007). Secretion of cerebellin CBLN1by granule cells is essentialfor the formation and stability of their synapses with Purkinje cells by bridging beta-neurexin and the glutamate receptor delta 2,GluRδ2(Hirai et al., 2005; Matsuda

et al., 2010; Uemura et al., 2010).CBLN1 can also stimulate the maturation of presynaptic boutons to match the size of the postsynaptic density (Ito-Ishida et al., 2012). Our results now show that expression of C1QL1 by inferior olivary neurons and of its receptor BAI3 by the target Purkinje cells is necessary for the development of climbing fiber /Purkinje cellsynapses.Thus the C1QL and CBLN subfamilies play similar and essential roles during brain development by promoting synaptogenesis between neurons that secrete them and target neurons that express their receptors. Their distinct and non-overlapping expression patterns ensure proper connectivity between different neuronal populations, suggesting that C1QL and CBLN subfamilies are part of the potential "chemoaffinity code" contributing to synapse specificity during circuit formation(Sanes and Yamagata, 2009; Sperry, 1963).

Interestingly these two subfamilies of complement C1Q-related proteins have distinct types of receptors, both at the structural and functional level: The BAI3 receptor is an adhesion-GPCR that binds C1QL proteins and controls RAC1 activation, while GluRδ2, the receptor for CBLN1, has a structure homologous to the glutamate ionotropic receptors and is coupled intracellularly to various signaling molecules such as PDZ proteins or the protein phosphatase PTPMEG (Yuzaki, 2011b). GluRδ2 becomes restricted to the parallel fiber/Purkinje cell synapses after P14 and is necessary for synapse formation and maintenance between parallel fibers and Purkinje cells. Its removal in genetically modified mice decreases the number of parallel fiber/Purkinje cell synapses,and consequently increases thesynaptic territory of climbing fibers (Uemura et al., 2007). Thus each excitatory input of Purkinje cells is characterized by a member of a specific C1Q-related subfamily that controls synaptogenesis on Purkinje cells through a different signaling pathway. Both GluRδ2 and BAI3 receptors are expressed early in Purkinje cells and remain highly expressed in the adult: if and how these two signaling pathways functionally interact to regulate synaptogenesis remains to be determined.

The subcellular localization of synapses between different types of inputs on a given target neuron is precisely controlled. For example, parallel fibers contact Purkinje cells on spines of distal dendrites whereas climbing fibers make their synapses on proximal dendrites. What regulates this level of specificity, essential for proper integration of signals in the brain, is poorly understood. Adhesion proteins have been involved, such as cadherin-9 for excitatory synapses in the hippocampus (Williams et al., 2011) or L1 family proteins for inhibitory synapses in cerebellar Purkinje cells(Ango et al., 2004). Studies of mutant mouse models, together with experiments involving lesions or modulation of activity, have demonstrated that parallel fibers and climbing fibers compete to establish their non-overlapping innervation pattern on cerebellar Purkinje cells (Cesa and Strata, 2009; Rossi and Strata, 1995). While parallel fiber/Purkinje cell synaptogenesis has already begun on the developing dendrites, a single climbing fiber starts translocating at P9 on the Purkinje cell primary dendrite (Hashimoto et al., 2009). These data suggest an active mechanism for the control of climbing fiber translocation and synaptic territory.*C1ql1* expression highly increases in inferior olivary neurons and becomes restricted to CFs in the olivocerebellar network starting at P7.Removing either C1QL1 from inferior olivary neurons or BAI3 from Purkinje cells,or misexpressing*C1ql1* in the cerebellum during postnatal development reduces theextent of the synaptic territory of CFs on their target Purkinje cells,showing that the secreted protein C1QL1 and its receptor the adhesion-GPCR BAI3 promote climbing fiber synaptic territory.The adhesion-GPCR BAI3 is also located at parallel fiber/Purkinje cell synapses and modulates the number of distal dendritic spines where those synapses are formed. Thusthe proper territory of innervation on Purkinje cellscould be controlled by the competition of excitatory afferents for a limited amount of BAI3 receptor sites. A deficient C1QL1/BAI3 pathway is not enough to prevent climbing fiber translocation (figures 2 and 4),and does not induce parallel fiber invasion of the climbing fiber territory (data not shown).Eph receptor

signaling has been shown to prevent invasion of the climbing fiber territory by parallel fibers, since its deficit induces spinogenesis and parallel fiber synaptogenesis in the proximal dendrites (Cesa et al., 2011). Thus climbing fiber synaptogenesis and translocation on Purkinje cells are controlled by different signaling pathways during development.

The C1QL/BAI3 signaling pathway might regulate synapse specificity in multiple neuronal populations that display segregation of synaptic inputs. In the hippocampus, mossy fibers from the dentate gyrus connect pyramidal cells on thorny excrescences close to the soma while entorhinal afferents form their contacts on distal portions of the dendrites.*C1ql3* is expressed by granule cells in the dentate gyrus and could thus control the segregation pattern of inputs on the dendritic tree of hippocampal pyramidal cells through interaction with the BAI3 receptor. Recently, the importance of secreted proteins in defining synapse specificity has also been highlighted in the invertebrate nervous system by the study of Ce-Punctin (Pinan-Lucarré et al., 2014). Thus the timely and restricted expression of secreted ligands and their interaction with receptors that regulate spinogenesis, synaptogenesis and synaptic territory constitute a general mechanism that coordinates the development of a specific and functional neuronal connectivity.

Experimental Procedures

All animal protocols and animal facilities were approved by the "Comité Régional d'Ethique en Expérimentation Animale" (#00057.01) and the veterinary services (C75 05 12).

cDNA and RNAi constructs

The shRNA sequences were5'tcgtcatagcgtgcatagg3' for CTL, 5'ggtgaagggagtcatttat3' for *Bai3* and 5'ggcaagtttacatgcaaca3'for *C1ql1*.They were subcloned under the control of the H1 promoter in a lentiviral vector that also drives *eGFP* expressionunder the control of PGK1 promoter(Avci et al., 2012).The *C1ql1 WT*cDNA construct (mouse clone #BC118980)was cloned into the lentiviral vector pSico (Addgene,Cambridge,MA, USA) under the control of the PGK1 promoter.The *eGFP* sequence of the original pSico was replaced by the cerulean sequence. The *C1ql1 Rescue* is a mutated form of *C1ql1 WT* with 3 nucleotide changes (T498C, A501C, C504T) that do not modify the amino acid sequence.

In vivo injections

Injections of lentiviral particlesin the cerebellum were performed in the vermis of anesthetized P7 Swiss mice at a 1.25mmdepth from the skull to target the molecular and Purkinje cell layers andat 1.120mmfor figure 5.Injections of lentiviral particles in the inferior olive were performed in anaesthetized P4 Swiss mice, on the left side of the basilar artery in the brainstem. Calibration of the injections showed thatthis procedure led to transduction of parts of the principal and dorsal accessory olive. 0.5µl to 1µl of lentivirus was injected per animal using pulled calibrated pipets.

Dendritic spine and synapse analysis

For each Purkinje cell, a dendritic segment of about 100 µm in length and in the distal part of the arborization or after the second branching point was considered. Dendritic spines were analyzed with the NeuronStudio software (version 9.92; Rodriguez et al. 2008). The spine head diameter corresponds to the minimal diameter of the ellipse describing the spine head, calculated in the xy axis. The spine length is the distance from the "tip" of the spine to the surface of the model. Minimum height was set to 0.5µm and maximum to 8µm.Synaptic contacts were analyzed using ImageJ customized macro. The CaBP and the vGluT1 objects found above a user-defined threshold were selected. Image calculator was used to extract the signal common to CaBP and vGluT1 images:the number and volume of these puncta were quantified with the 3D Object counter plugin from ImageJ.The size of presynaptic vGluT2 clusters was analyzed using the ImageJ plugin 3D object counter. Bin number of vGluT2 cluster intersection was assessed using the Advanced Scholl analysis plugin from ImageJ.

Statistical analysis

Data generated with NeuronStudio or ImageJ were imported in GraphPad Prism for statistical analysis. Data were analyzed by averaging the values for each neuron in each condition. Valuesare given as mean ± S.E.M. Student *t* test or One way ANOVA followed by Newman-Keuls *posthoc* test were performed for comparison of two or more samples respectively. When distribution did not fit the Normal law (assessed using Graphpad Prism), Mann–Whitney *U*test or One-way ANOVA followed by Kruskal-Wallis *posthoc* test were used.Two way ANOVA followed by Bonferroni *posthoc* test was performed for the analysis of bin number of vGluT2. *$p < 0.05$; ** < 0.01; ***$p < 0.001$.

Supplemental material and methods (cerebellar mixed cultures, RTqPCR, *in situ* hybridization, immunohistochemistry, image acquisition and electrophysiology) are available online.

Author Contributions

F.S., S.S. and P.I. designed the experiments. S.S., K.I, F.B., I.G.C., M.T. performed experiments. G.V. provided critical reagents. All authors discussed the data. F.S., S.S. and P.I. wrote the manuscript.

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Figure Legends

Figure 1. Developmentally regulated expression of the *Bai3* and*C1ql*genesin the mouse brain.

(A)*In situ* hybridization experiments were performed using probes specific for*Bai3*, *C1ql1* and *C1ql3* on coronal (left) and sagittal (right) sections of mouse brain taken at postnatal day 0 (P0), P7 and adult. Ctx, cortex; DCN, deep cerebellar nuclei; DG, dentate gyrus; Hp, hippocampus; IO, inferior olive; LA, lateral amygdala; PC, Purkinje cell.Scale bars, 500µm; each scale bar applies to the whole column.**(B)**Expression of *Bai3* and *C1ql1* was assessed at different stages of mouse brain development using quantitative RT-PCR on mRNA extracts from brainstem and cerebellum (E17: embryonic day 17; P0 to P14: postnatal day 0 to 14). Expression levels are normalized to the *Rpl13a* gene. N=3 samples per stage. (See also Figure S2).

Figure 2. The adhesion-GPCR BAI3 promotes synaptogenesis and the innervation territory of climbing fibers on Purkinje cells.

(A,B) Defects in climbing fiber synapses were assessed at P21 after stereotaxic injections at P7 of recombinant lentiviral particles driving expression of shRNA against *Bai3* (shBAI3) or control shRNA (shCTL).Immunostaining for vesicular glutamate transporter 2 (VGluT2) was used to label specifically climbing fiber synapses on transduced PCs (eGFP positives), A, Representative images of vGluT2 extension.Pial surface:white dashed line. Scale bar, 40µm. B, Representative images of vGluT2 cluster morphology. Scale bar, 10µm.**(C)**The extension of vGluT2 clusters relative to Purkinje cell height, their mean number and volume were quantified using Image J. N≥22 cells,N=3 animals per condition. Data are presented as mean ± SEM; unpaired Student *t* test or Two way ANOVA followed by Bonferroni *posthoc* test, *p < 0.05; **p < 0.01; ***p < 0.001. **(D)**Electrophysiological recordings ofP18 to P23 Purkinje

cells transduced with recombinant lentiviral particles driving expression of either shBAI3 or shCTL. Climbing fiber-mediated whole cell currents are shown in the left panel. Averages of five stimuli for two representative cells are shown. Traces were recorded at – 10 mV following CF stimulation. Total CF-mediated EPSCs were quantified and plotted in the bar graph shown in the right panel. Bars represent mean ± SEM.Unpaired Student *t* test, *p < 0.05. (See also Figure S3A and S5A).

Figure 3. The adhesion-GPCR BAI3 promotes spinogenesis and parallel fiber synaptogenesisin Purkinje cells.

(A) Reduced spine density in distal dendrites of Purkinje cells after*in vivo*knockdown of *Bai3* using stereotaxic injections of lentiviral particles in the vermis of P7 mice. Effects of shBAI3 or shCTL expression were visualized at P21 on transduced (eGFP positive) PCs. Scale bars, 5µm.**(B)**PF-ESPCs recorded in Purkinje cells (P18 to P23)after stereotaxic injections of lentiviral particlesat P7. Averaged traces recorded at maximum stimulus intensity are shown for one representative cell per condition (control: PF-shCTL, left;BAI3 knockdown:PF-shBAI3, right). Input/output curves obtained for both conditions are significantly different (right panel: Kolmogorov-Smirnov test, $p < 0.001$). Data are normalized to the mean value of responses elicited by the minimum stimulus intensity (-29.63 pA ± 9.92 for shCTL and -32.54 pA ± 10.57 for shBAI3) and are plotted as mean ± SEM against stimulus intensity (shCTLblack square, n = 9 and shBAI3 grey diamond, n = 10).**(C)**Cerebellar mixed cultureswere transduced at DIV4 with recombinant lentiviral particles driving expression of GFP together with shBAI3 or control shCTL. Dendritic spines and parallel fiber synapses in transduced Purkinje cells (GFP positive) were imaged at DIV14 afterimmunostaining for calbindin (CaBP) and vesicular glutamate transporter 1 (VGluT1). Scale bar: 5µm.**(D)**Quantitative assessment of the number and morphology of Purkinje cell spines was performed using the NeuronStudio software. N≥31 cells per condition, 3 independent

experiments (Data are presented as mean± SEM; unpaired Student *t* test, *p < 0.05; ***p < 0.001). **(E)**Quantitative assessment of the number and size of vGluT1 synaptic contactsin DIV14 Purkinje cells was performed using ImageJ. N≥30 cells per condition, 3 independent experiments (Data are presented as mean± SEM; unpaired Student *t*test and Mann-Whitney *U* test, respectively, *p < 0.05; ***p < 0.001). (See also Figure S3A, S4 and S5).

Figure 4. The C1QL1 protein from inferior olivary neurons promotes climbing fiber/Purkinje cell synaptogenesis.

(A) Defects in climbing fiber/Purkinje cell synapses were assessed at P14 after *C1ql1* knockdown. Stereotaxic injections of recombinant lentiviral particles driving expression of a shRNA against *C1ql1* (shC1QL1), a control shRNA (shCTL) or shC1QL1together with a*C1ql1*rescuecDNA were performed in the inferior olive of P4 mice. Immunostaining for vesicular glutamate transporter 2 antibody (VGluT2) was used to visualize climbing fiber synapses.eGFP positive CFs correspond to transduced IONs. Scale bars, left panel, 20µm; right panel, 10µm. **(B)** Extension of climbing fibers (eGFP) or ofclimbing fiber synapses (vGluT2) relative to Purkinje cell height, as well as the number of climbing fiber synapses were quantified using Image J. N=4-8 animals and N≥95 CFs per condition. Data are presented as mean ± SEM; One-way ANOVA followed by Kruskal-Wallis posthoc test or Dunn's test, *p < 0.05; **p < 0.01; ***p < 0.001. **(C)** Top panel, climbing fiber-induced EPSCs recorded in Purkinje cells located in the target zone of virally transduced climbing fibers (cf. text). Bottom panel, summary bar graphs showing the averaged peak amplitude of CF EPSCs for each condition. Bars represent mean ± SEM values.Mann Whitney *U* test, *p < 0.05.(See also Figure S6).

Figure 5. Misexpression of *C1ql1*reducesthe synaptic territory of climbing fibers on Purkinje cells.

C1ql1 misexpression in the cerebellum was performed using stereotaxic injections in the vermis of P7mice of lentiviral particles driving the expression of green fluorescent protein (eGFP) alone or together with C1QL1 (C1QL1 WT). Climbing fiber extensionwas imaged at P14after immunostaining for vGluT2 (CF synapses) and CaBP(entire PC). N=6 animals per condition. Data are presented as mean± SEM; unpaired Student *t* test with Welch's correction, ***p < 0.001. Scale bars, 40 µm.

Figure 6. Transient C1QL1 secretion in the cerebellumpromotes Purkinje cell spinogenesis.

(A) The role of cerebellar C1QL1 was assessed in mixedcultures using an RNA interference approach. Neurons were transduced at DIV4 with recombinant lentiviral particles driving expression of control shRNA (shCTL) or shC1QL1, or a mixture of driving either shC1QL1and wild-type *C1ql1* (knockdown condition),orshC1QL1 and *rescue C1ql1* cDNA (control condition). High-resolution confocal imaging was performed at DIV14 after immunostainingfor calbindin (CaBP) and vesicular glutamate transporter 1 (VGluT1, specific for parallel fiber synapses). Scale bar, 5µm.**(B)**Effects of *C1ql1* knockdown on spine density, head diameter and length, as well as on the number of vGluT1 synaptic contacts were quantified. N≥30 cells, 3-4 independent experiments. Data are presented as mean± SEM; One-way ANOVA followed by Newman-Keulsor Kruskal-Wallis*posthoc* test, *p < 0.05; **p < 0.01; ***p < 0.001. Spine density was significantly reduced in all conditions when compared to the shCTL condition. See also Figure S3B.

Figure 7. The modulation by C1QL1 of Purkinje cell spinogenesisdepends on normal levels of the BAI3 receptor

(A) The functional interaction between C1QL1 and BAI3 was assessed by simultaneous reduction of their expression in cerebellar cultures using an RNA interference approach.

Neurons were transduced at DIV4 with a mixture of recombinant lentiviral particles driving either shC1QL1 and shBAI3 (double knockdown) or shBAI3 and shCTL (*Bai3* knockdown alone) or shC1QL1 and shCTL (*C1ql1* knockdown alone) or double amounts of shCTL.Analysiswas performed using high-resolution confocal imaging at DIV14 after immunostainingfor calbindin (CaBP). Scale bar, 5µm. **(B)** Quantitative analysis of spine density performed using Neuron Studio. N≥30 cells, 3-4 independent experiments(Data are presented as means ± SEM; One-way ANOVA followed by Newman-Keuls*posthoc* test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Spine density was significantly reduced in all conditions when compared to the shCTL condition.