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Behavioral training rescues motor deficits in Cyfip1 haploinsufficiency mouse model of autism spectrum disorders

Sven O. Bachmann1, Monika Sledziowska1, Ellen Cross1, Shireene Kalbassi1, Sophie Waldron1, Fangli Chen2, Adam Ranson2,3 and Stéphane J. Baudouin1

Abstract
Deletions in the 15q11.2 region of the human genome are associated with neurobehavioral deficits, and motor development delay, as well as in some cases, symptoms of autism or schizophrenia. The cytoplasmic FMR1-interacting protein 1 (CYFIP1) is one of the four genes contained within this locus and has been associated with other genetic forms of autism spectrum disorders (ASD). In mice, Cyfip1 haploinsufficiency leads to alteration of dendritic spine morphology and defects in synaptic plasticity, two pathophysiological hallmarks of mouse models of ASD. At the behavioral level, however, Cyfip1 haploinsufficiency leads to minor phenotypes, not directly relevant for 15q11.2 deletion syndrome or ASD. A fundamental question is whether neuronal phenotypes caused by the mutation of Cyfip1 are relevant for the human condition. Here, we describe a synaptic cluster of ASD-associated proteins centered on CYFIP1 and the adhesion protein Neuroligin-3. Cyfip1 haploinsufficiency in mice led to decreased dendritic spine density and stability associated with social behavior and motor learning phenotypes. Behavioral training early in development resulted in alleviating the motor learning deficits caused by Cyfip1 haploinsufficiency. Altogether, these data provide new insight into the neuronal and behavioral phenotypes caused by Cyfip1 mutation and proof-of-concept for the development of a behavioral therapy to treat phenotypes associated with 15q11.2 syndromes and ASD.

Introduction
Autism spectrum disorders (ASD) are defined by persistent deficits in social communication and social interaction accompanied by restricted, repetitive patterns of behavior, interests, or activities. In addition to these core symptoms, more than 70% of individuals with ASD have co-occurring medical, developmental, or psychiatric conditions1. An extensive variety of impairments affecting gross and fine motor functions affect more than 79% of individuals with ASD1. Despite the frequency of motor anomalies, studies on the underlying pathophysiology of motor deficits and their treatment remain limited.

Deletions in the 15q11.2 region of the human genome are associated with neurobehavioral deficits, and motor development delay and, in about 30% of individuals, symptoms of autism7. The 15q11.2 locus contains four genes, tubulin gamma complex-associated protein 3 (TUBGCP3), cytoplasmic FMR1 interacting protein 1 (CYFIP1), and non-imprinted in Prader-Willi/Angelman syndrome 1 and 2 (NIPA1/2), and is also associated with larger 15q11–q13 type I deletions causing Prader-Willi or Angelman Syndromes3. Microdeletions of 15q11.2 correlated with decreased TUBGCP3, CYFIP1, and NIPA1/2 mRNA levels in lymphoblastoid cells derived from patients with Prader-Willi syndrome4. The four genes in the 15q11.2 locus were shown to impact on the behavioral
symptoms of patients with 15q11.2 deletion or Prader-Will syndrome. In addition, decreased CYFIP1 mRNA levels were also found in leukocytes obtained from ASD diagnosed patients with Fragile X and in patients diagnosed with autism and carrying a deletion of SH3 and multiple ankyrin repeat domains 2 (SHANK2) gene.

Amongst the genes within region 15q11.2, CYFIP1 is of particular interest due to its role in the control of actin dynamics\textsuperscript{5,6} and protein translation\textsuperscript{7}. This regulation occurs through its interaction with the Wiskott–Aldrich syndrome protein family verprolin-homologous protein (WAVE) regulatory complex (WRC)\textsuperscript{5,6}, and fragile X mental retardation protein (FMRP)\textsuperscript{7}, respectively. Hippocampal neurons with Cyfip1 haploinsufficiency (Cyfip1\textsuperscript{HET}) show defects in dendritic spine morphogenesis and increased metabotropic glutamate receptor-induced long-term depression\textsuperscript{8,9}, two phenotypes consistently found in mouse models of ASD and in particular Fragile X syndrome\textsuperscript{10–12}. Importantly, analyses of post-mortem tissue of individuals with idiopathic and syndromic forms of ASD also showed alteration of dendritic spine morphology\textsuperscript{11}. These results show that the effects of deletion of Cyfip1 resemble the cellular phenotypes found in other models of ASD. However, mice heterozygous for Cyfip1 (Cyfip1\textsuperscript{HET}) show mild behavioral phenotypes\textsuperscript{8,13}. It is, therefore, possible that CYFIP1 function at synapses is not relevant for ASD or that the behavioral phenotyping of Cyfip1\textsuperscript{HET} mice was not conducted extensively enough.

Within the WRC, CYFIP1, and Abi interactor proteins 1–3 (ABI1–3) form an interaction surface that enables the interaction with proteins containing a WRC interacting receptor sequence (WIRS). The coding sequence of synaptic adhesion proteins Neuroligin-3 contains a WIRS and is therefore predicted to interact with CYFIP1 and

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![Fig. 1 Binding between Neuroligin-3 and Cyfip1 in Pvalb-expressing neurons.](image)

**A** Western blot analysis showing that Neuroligin-1, 2, and 3 (NL1, 2, and 3) and Cyfip1 are detected in proteins co-immunoprecipitated with Neuroligin-3 in wild-type striatum (St), cerebellum (Cb), and cortex (Cx). Note that FMRP is not detected in co-immunoprecipitated proteins. **B** Western blot analysis showing that Neuroligin-2 (NL2), Cyfip1, and FMRP are detected in proteins co-immunoprecipitated with Neuroligin-3 in striatum (St), cerebellum (Cb), and cortex (Cx) of Nlgn3\textsuperscript{KO} Pvalb\textsuperscript{Cre} mice. Note that Neuroligin-1 was not detected in co-immunoprecipitated proteins. **C** Two-step cluster analysis of proteins co-immunoprecipitated with Neuroligin-3 in wild-type mice VNO, Nlgn3\textsuperscript{KO} Pvalb\textsuperscript{Cre}. **D** Bar graphs show the number of proteins per cluster and per anatomical region. **E** Details of the anatomical region (VNO (V), striatum (S), or cerebellum (C)) and cluster in which proteins coded by genes associated with ASD and co-immunoprecipitated with Neuroligin-3 are detected.
 ABI 1–3. The genes coding for these proteins have been associated with ASD in humans14–19. A putative interaction between Neurulgin-3, CYFIP1, and FMRP would point towards a possible common pathway linking ASD-associated proteins at synapses. Mice lacking Neurulgin-3 or FMRP show defects of social behavior and motor learning deficits correlated20–25 with alterations of dendritic spine stability26,27. Based on this predicted interaction, we hypothesized that Cyfip1Het mice also show defects in social and motor behaviors. This study examines these possibilities by immunoprecipitation paired with mass-spectrometry, analysis of dendritic spine plasticity in vivo and behavioral analysis. Based on the results obtained, we investigated the possibility rescuing Cyfip1Het mice motor learning phenotype using a behavioral approach.

Results

The interaction between Neurulgin-3 and CYFIP1 was investigated using immunoprecipitation of Neurulgin-3 in striatum, cerebellum, and cortex, followed by western blot analysis. Neurulgin-3 co-immunoprecipitates Neurulgin-1 and 2, two previously characterized interactors of Neurulgin-328, and CYFIP1 (Fig. 1a). CYFIP1 was detected in the three different brain regions indicating that the interaction between Neurulgin-3 and CYFIP1 is not restricted to a particular brain region. Note that FMRP was not detected in these samples. Further investigations revealed that CYFIP1 was also present in Neurulgin-3 immunoprecipitates from synaptosomes but again FMRP was not (Supplementary Figure 1a). Neurulgin-3 is expressed in neurons but also in astrocytes29. To determine if the interaction between Neurulgin-3 and CYFIP1 occurs in neurons, immunoprecipitations were performed on brain samples originating from mice expressing Neurulgin-3 only in Pvalb-expressing neurons (Nlgn3KO-PvalbCre) as we previously showed that re-expressing Neurulgin-3 in these cells was sufficient to restore the ASD-related deficits in Nlgn3 knockout mice30. Neurulgin-3 neuronal co-immunoprecipitated proteins from the three brain regions contained Neurulgin-2 and CYFIP1, indicating that indeed interaction between these proteins occurs in neurons and is not grossly influenced by the regional localization of Pvalb-expressing neurons (Fig. 1b and supplementary Fig. 1b). Interestingly, proteins from Nlgn3KO-PvalbCre, co-immunoprecipitated with Neurulgin-3, did not contain Neurulgin-1 but did contain FMRP, suggesting that the identity of the proteins interacting with the Neurulgin-3/CYFIP1 complex vary depending on the cell type.

Studies suggest that CYFIP1’s interaction with FMRP and WRC is mutually exclusive7. To determine if the Neurulgin-3/CYFIP1 complex interacts exclusively with FMRP or also contains components of the WRC we performed an unbiased screen of Neurulgin-3 co-immunoprecipitated proteins using mass-spectrometry. The synapse-specific interactome of Neurulgin-3 was investigated, in vivo, in Nlgn3KO-PvalbCre mice tissue (N = 3, supplementary Table 1), and in tissue expressing Neurulgin-3 but lacking synapses (N = 3, supplementary Table 2), as a control. The so-called olfactory ensheathing cells (OEC) populating the vomeronasal organ (VNO) fulfill these criteria as they are known to express Neurulgin-330, but are entirely devoid of synapses. Note that, biochemical analysis and in situ hybridization demonstrated that Neurulgin-3 is expressed in non-neuronal cells in the VNO, confirming its expression in OEC (supplementary Fig. 2a–d). To determine proteins specifically associated with Neurulgin-3 in Pvalb-expressing interneurons, we performed co-immunoprecipitation of Neurulgin-3 coupled with mass-spectrometry analysis and included co-immunoprecipitation of the VNOs of wild-type (N = 3, supplementary Table 2) and Nlgn3KO mice (N = 2) to our analysis to control for non-neuronal co-immunoprecipitated proteins and unspecific binding of the Neurulgin-3 antibody, respectively (Supplementary Tables 1 and 2). We used hierarchical cluster analysis of co-immunoprecipitated proteins in wild-type mice VNO or Nlgn3KO-PvalbCre mice striatum or cerebellum but not in Nlgn3KO mice VNO to determine the optimal number of clusters in our dataset, which was found to be three. On this basis, follow-up two-step cluster analysis showed that clusters 1, 2, and 3 contained 42.6%, 42.1%, and 15.3% of co-immunoprecipitated proteins, respectively (Fig. 1c). Analysis of the number of co-immunoprecipitated proteins contained in each cluster revealed that cluster 1 proteins almost uniquely originated from the striata and cluster 2 proteins originated from the three anatomical regions (Fig. 1d). Interestingly, cluster 3 was mostly composed of proteins co-immunoprecipitated from striata and/or cerebella but not VNOs, suggesting the existence of proteins interacting with Neurulgin-3 specifically in neurons. Consistent with Neurulgin-3 synaptic localization in neurons, excitatory (PSD93 and 95 and synapse-associated protein 97 and 102), and inhibitory synaptic proteins (Neurulgin-2 and Neurulgin-4 and Gephyrin) were co-immunoprecipitated only in Pvalb-expressing neurons independently of the brain region (Supplementary Table 3). As FMRP, WAS protein family member 1 (WASF1) and ABI1 were detected in proteins immunoprecipitated by Neurulgin-3. FMRP was detected in Nlgn3KO-PvalbCre mice cerebella and striata, whereas WASF1 and ABI1 were only detected in striata (Supplementary Table 3). These results suggest that binding between Neurulgin-3 and CYFIP1 and WRC is influenced by the localization of the Pvalb-expressing cells.
Interestingly, the protein products of genes reliably associated with syndromic and non-syndromic ASD (with an SFARI gene score of 1, 2, or 3) were also co-immunoprecipitated only in Pvalb-expressing interneurons, independently of the brain region (Fig. 1e). Altogether, these results show that Neuroligin-3 and CYFIP1 interact in neurons in vivo and that the identity of the proteins they interact with is influenced by the regional localization of the neurons. Moreover, they suggest that CYFIP1 may interact with other ASD-associated proteins at excitatory and inhibitory synapses, either directly or indirectly via its binding to Neuroligin-3 (Supplementary Fig. 1b).

Mice lacking Fmr1 or Nlgn3 show behavioral phenotypes associated with the core symptoms of ASD, including defects in social behavior, but also with some of its comorbidities (e.g. hyperactivity, alteration of motor learning). We hypothesized that since CYFIP1 interacts with both Neuroligin-3 and FMRP, the behavioral phenotype should be similar across the three models. A complete absence of Cyfip1 is embryonic lethal, therefore we used a Cyfip1HET mouse model. Cyfip1HET mice showed an absence of interest for social cues compared to their Cyfip1WT littermates (Fig. 2a) without deficits in social dominance (courtship vocalization test, Fig. 2b) or repetitive behaviors (marble burying test, Fig. 2c). In addition, Cyfip1HET mice showed motor learning deficits, as indicated by the absence of an improved performance in the rotarod test, compared to their Cyfip1WT littermates (Fig. 2d), but no change in basal motor activity (Fig. 2e, f). The biochemical characterization of Cyfip1HET mice showed a significant decrease of CYFIP1 protein expression levels in motor and frontal cortex and in the hippocampus but no change in other brain regions or in the periphery (Supplementary Fig. 3). Cyfip1HET mice phenotype is consistent with the region-specific decrease of CYFIP1 protein expression since frontal and motor areas of the cortex are involved in the control of social and motor functions. In addition, the behavioral phenotype of Cyfip1HET mice is also consistent
with the association between CYFIP1 and 15q11.2 deletion syndrome and ASD in humans, therefore validating the use of the mouse model for ASD studies.

Cyfip1HET hippocampal neurons show deficits in dendritic spine maturation in vitro and ex vivo, a converging cellular phenotype between syndromic and non-syndromic genetic mouse models of ASD. Decreased dendritic spine maturation can be explained by defects in dendritic spine formation and/or dendritic spine instability. To investigate these two possibilities, we crossed Cyfip1HET mice with mice expressing green fluorescent proteins in a subset of neurons (refer to Material and methods) and imaged dendritic spines of cortical neurons ex vivo and in vivo in adult male mice. Immunohistology experiments demonstrated that Cyfip1HET mice showed decreased dendritic spine density in neurons from motor cortex but not from the V1 area of the visual cortex, compared to their Cyfip1WT littermates (Fig. 3a). Two-photon imaging to track dendritic spines in the motor cortex in vivo revealed an increased formation and elimination of dendritic spines over time, in Cyfip1HET mice, compared to Cyfip1WT littermates (Fig. 3b, c). Since increased rotarod performance correlates with increased formation of dendritic spines, we hypothesized that, in addition to increased dendritic spine instability, Cyfip1HET mice show defects in dendritic spine formation. As previously demonstrated, motor training increased the formation of dendritic spines by about three-fold in Cyfip1WT mice without affecting the number of eliminated spines (Fig. 3d). Despite their lack of

Fig. 3 Cyfip1 haploinsufficiency leads to decreased stability of dendritic spines without affecting their formation. a Dendrites of GFP-expressing neurons from the motor cortex (M1), visual cortex (V1), and hippocampus (CA1 and CA3) of Cyfip1WT and Cyfip1HET mice. Scale bars represent 10 μm. Quantification of spine density of Cyfip1WT and Cyfip1HET dendrites. b Two images of the same dendrite of a Cyfip1WT mice motor neuron taken at an interval of 2 days showing the newly formed dendritic spines (arrows). Scale bars represent 2 μm. c Quantification of formed and eliminated dendritic spines in motor cortices of Cyfip1WT and Cyfip1HET mice. d Quantification of formed dendritic spines in motor cortices of Cyfip1WT and Cyfip1HET mice before (B) and after rotarod training (R). All values are represented as mean ± SEM. In (b) and (d), statistical significance was tested by ANOVA followed by Bonferroni post-hoc test. **P ≤ 0.01

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increased performance on the rotarod, Cyifp1<sup>HET</sup> mice showed an increased number of newly formed dendritic spines upon motor training similar to that of their Cyifp1<sup>WT</sup> littermates (Fig. 3d). Note that rotarod training had no effect on dendritic spine elimination in both genotypes. These results show that while Cyifp1<sup>HET</sup> does not impact on the ability to form new spines it leads to an increased dendritic spines instability.

Given that Cyifp1<sup>HET</sup> mice show a similar ability to form new spines upon training as Cyifp1<sup>WT</sup> mice, we hypothesize that training Cyifp1<sup>HET</sup> mice during development, before the appearance of the behavioral phenotype, would be sufficient to rescue their motor learning deficits. The earliest time point in the development animals complied with the motor learning protocol was postnatal day 40 (P40), when Cyifp1<sup>WT</sup> mice show reliable decreased latency to fall from an accelerating rod over successive trials (Fig. 4a). At P40, Cyifp1<sup>HET</sup> mice showed a decreased latency to fall from the accelerating rod over successive trials (Fig. 4b). These results indicate that Cyifp1<sup>HET</sup> mice motor learning deficits develop after P40. To test our hypothesis we trained Cyifp1<sup>WT</sup> and Cyifp1<sup>HET</sup> mice at postnatal days 40, 50, and 51 using the same rotarod protocol (in the following referred to as 'trained' mice) and tested for their motor behavior at P60. Trained Cyifp1<sup>WT</sup> mice showed an increased latency to fall from an accelerating rod at trials 1 and 2 and no difference at the subsequent trials compared to untrained adult wild-type mice, indicating that, as previously demonstrated, motor training increased the basal motor performance of wild-type mice (Fig. 4c). Trained Cyifp1<sup>HET</sup> mice showed a similar latency to fall from an accelerating rod from trials 1 to 4 and subsequently increased latency to fall compared to untrained Cyifp1<sup>HET</sup> mice, suggesting that sustained motor training during the development is sufficient to alleviate the motor deficits of adult Cyifp1<sup>HET</sup> mice (Fig. 4d).

**Discussion**

Three important conclusions can be drawn from this study. First, we found that CYFIP1 binds to Neuroligin-3.
at synapses and, directly or indirectly, to other proteins associated with ASD. Second, we showed that *Cyfip1*<sup>HET</sup> is associated with alterations of dendritic spine stability, but not formation, and defects in social behavior and motor learning. Finally, based on these findings we designed a behavioral approach that alleviated the motor learning phenotype of *Cyfip1*<sup>HET</sup> mice.

**Dendritic spine defects as a converging phenotype in ASD**

Previous work with genetic mouse models suggested that the different genetic mutations associated with ASD target a small set of molecular pathways, leading to a deficit in dendritic spine morphogenesis<sup>10,11</sup>. Our study is the first to provide direct evidence to support this hypothesis. Importantly, defects in dendritic spine morphology are also reported in human post-mortem studies, showing that this pathophysiological feature is one of the few conserved between mouse models and humans.

When studied in isolation, the impact of specific monogenic genetic risk factors for ASD is limited by the frequency of the mutations in the human population, which is, in general, very low<sup>36</sup>. Our results demonstrate that some of these risk factors are in fact directly connected, such that they are likely to form a single molecular pathway in which dysfunction can cause specific forms of ASD. The frequency of such molecular alterations is likely to be equivalent to the sum of the frequency of the genetic risks, thus accounting for a large number of individuals with ASD. It remains possible that independent pathways regulating dendritic spine formation and function may alter the composition of this cluster of ASD proteins, suggesting that, not all cases of ASD associated with such changes are present, can be attributed to a single cause. Nevertheless, this finding is likely to provide a framework to address the genetic heterogeneity associated with ASD.

As such, analysis of dendritic spine morphology or density cannot be conveniently used as a biomarker in the human population, as it requires access to brain tissue. In this context, our findings are more likely to represent a step towards a better definition of some of the biological basis of ASD, which in turn may lead to the identification of biological markers. Indeed, dendritic spines are the postsynaptic docking compartment for a large number of excitatory synapses and decrease in their number and stability is likely to have a direct consequence on the number of synaptic inputs a neuron receives and, by extension, a brain region receives. Such connectivity between brain regions can be quantified in mice and humans using diffusion tensor imaging. Using *Cyfip1*<sup>HET</sup> mouse model, and by extension, any genetic model associated with the ASD protein cluster could, therefore, help to better understand the complex relationship between brain region-specific defects in dendritic spine stability and brain-wide connectivity providing an experimental framework to identify biological markers for specific behavioral phenotypes.

**Predicted functional consequences associated with the ASD cluster of proteins**

Aberrant signaling through type 1 metabotropic glutamate receptors 1 and 5 (mGluR1 and 5), resulting in defects in long-term synaptic depression (LTD), is a pathophysiological hallmark of genetic mouse models of ASD<sup>37</sup>. Interestingly, mutations in genes associated with the proteins co-immunoprecipitated with Neurroligin-3, including *Neurroligin-3* itself<sup>38,39</sup> (but see ref. 40), *CYFIP1*

*FMRP*<sup>40</sup>, *MeCP2*<sup>41</sup>, *Synaptic Ras GTPase-activating protein 1* (encoded by the *Syngap1* gene)<sup>42</sup>, and the beta 3 subunit of Type A gamma-aminobutyric acid receptors (encoded by the *Gabrb3* gene)<sup>43</sup>, lead to such phenotypes. These results indicate that in addition to regulating dendritic spine morphogenesis, this cluster of proteins might be involved in regulation of mGluR1/5 function and control of LTD.

At synapses, mGluR1/5 binds directly to Homer proteins, which links the receptors to the endoplasmic reticulum (ER), via interactions with PI 3-kinase enhancer (PIKE) and inositol 1,4,5-trisphosphate receptors (IP3Rs), or to ionotropic glutamate receptors, via SH3 and multiple ankyrin repeat domains (Shank) proteins<sup>44</sup>. Our data show that type 1 mGluR, and mGluR1 specifically, are reliably co-immunoprecipitated with Neurroligin-3 in cerebellar but not striatal *Pvalb*-expressing cells (Supplementary Table 3). Moreover, along with mGluR1 we also reliably detected PIKE (encoded by the *Agap3* gene) and IP3R1 (encoded by the *Itpr1* gene) but not Shank proteins or ionotropic receptors (Supplementary Table 3).

Interestingly, FMRP has been shown to inhibit PIKE and therefore type 1 mGluR downstream signaling<sup>45</sup>. These results raise the question of a potential involvement of CYFIP1 in regulating type 1 mGluR downstream signaling. It is possible that Neurroligin-3, FMRP, and CYFIP1 are expressed in different subcellular compartments of the neurons and, therefore, do not aggregate in a single cluster. Nevertheless, these results suggest that Neurroligin-3, FMRP, and CYFIP1 are involved in localizing mGluR1 and the ER at the synapse. The release of calcium from the ER to the cytoplasm is regulated by mGluR1 and essential for the induction of LTD<sup>46</sup>. Based on our results, we can further speculate that defects in mGluR-LTD observed in genetic mouse models for ASD result from an inappropriate localization of the mGlur/ER complex at the synapse, a hypothesis that remains to be experimentally tested. Importantly, this mechanism might be cell or type 1 mGluR specific as we did not reliably detect type 1 mGluR in proteins co-immunoprecipitated with Neurroligin-3 in the striatum.
Behavioral treatment to alleviate ASD core and comorbid symptoms

About 42% of individuals carrying 15q11.2 deletions show motor delay\(^2\). Motor abnormalities and delay in motor development are common comorbid symptoms in more than 70% of individuals with ASD\(^3\). In this study, we found that adult Cypbfp1\(^{HET}\) mice show defects in motor learning. Note that we have not fully characterized the motor learning deficits and it is possible that a less challenging paradigm, with longer intertrial intervals for example, would find milder or no phenotype in Cypbfp1\(^{HET}\) mice. Nevertheless, along with Cypbfp1\(^{HET}\) mice, other mouse models for ASD show motor phenotypes\(^4\), suggesting that, to a certain extent, mouse models of ASD replicate motor abnormalities found in humans. Importantly, we found that training Cypbfp1\(^{HET}\) mice early in the development is sufficient to alleviate the motor learning phenotype in adult mice. These results demonstrate that the motor learning phenotype of Cypbfp1\(^{HET}\) mice develops over time, appearing between young adult and adult stages, and is reversible using behavioral training approaches. As future studies, it would be interesting to know if dendritic spine phenotypes follow the similar developmental trajectories and sensitivity to behavioral training. These results provide important insights for the treatment of motor impairment in ASD in humans. In humans, motor impairments can be detected early in the development alongside the diagnosis of ASD. Our results suggest that early behavioral intervention, targeted at stimulating motor function, might help in preventing motor impairment arising later in life.

Materials and methods

Animals

All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 (amended 2012). Nlgn3\(^{K0}\) mice\(^48\) were crossed with Omp\(^{Cre}\) mice (JAX: #006668\(^49\)), where part of the Omp gene was replaced by Cre recombinase. Nlgn3\(^{K0}\) mice were also crossed with Pvalb\(^{LoxPl}\) mice, expressing Cre recombinase under the Pvalb promoter (JAX:01732050). The Cypbfp1\(^{tm2a}\) (EUCOMM)\(^{Wtsi}\) (EUCOMM) mouse line was either bred with BL6 mice to obtain Cypbfp1\(^{HET}\) and Cypbfp1\(^{WT}\) mice or crossed with Tg(Thy1-EGFP)Mirs/J (JAX: # 007788\(^51\)) to obtain Thy1-EGFP-Cypbfp1\(^{Het}\) and Thy1-EGFP-Cypbfp1\(^{WT}\) mice. Mice were kept on a 12 h light/dark cycle with free access to food and water. All behaviors were assessed during the light cycle and after mice were habituated for 30 min to the testing room.

Biochemistry

Brain tissue from adult mice was sliced in 1.5–2 mm sections and micro-dissected prior to snap freezing and storage at −80 °C. 10 µl of lysis buffer (1% SDS, 10 mM hepes, protease inhibitors, phosphatase inhibitors, 1 mM NaF, 1 mM NaVO\(_4\)) was used per 1 mg of tissue. Using Laemmli buffer samples were run on SDS–PAGE gels (NuPAGE Novex 4–12% Bis–Tris, Protein gels, Invitrogen) and transferred on nitrocellulose membrane. Immunoblotting was performed with antibodies raised against CYFIP1 (anti-CYFIP1 ab156016, Abcam), β-III Tubulin (801201, BioLegend), and GAPDH (83956, Abcam). Blots were developed using ECL (Santa Cruz and Advansta). Blots were digitally acquired by ChemiDoc (Biorad) and quantified using Image Lab Software (Biorad).

For synaptosome fractionation, mice were culled by cervical dislocation, and the brains were immediately dissected and homogenized in SynPer (Sigma-Aldrich) containing protease cocktail inhibitors (Sigma-Aldrich) and phosphatase inhibitors (10 mM NaF, 1 mM NaVO\(_4\)). Homogenized tissues were then centrifuged to give pellets, resuspended in SynPer to obtain nuclear fractions, and supernatants (i.e. homogenates). Part of the supernatants was further centrifuged to give pellets, further resuspended in SynPer\(^*\) to give synaptosome fractions and cytosolic fractions in supernatants. The samples were then immediately processed for western blot analysis, or immediately used for immunoprecipitation experiments.

For immunoprecipitation, tissues were dissected and then homogenized in 100 ml of lysis buffer for each 10 mg of tissue (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 10 mM NaF, 1 mM Na3VO\(_4\), protease inhibitor cocktail (Sigma-Aldrich)). The samples were centrifuged at 14,000 × g for 10 min at 4 °C and the supernatant containing the proteins was removed. The ethanol was removed from Sepharose beads coated with Protein A (GE Healthcare Life Sciences) by two washes with ice-cold phosphate-buffered saline, at 2000 × g for 2 min. The supernatant was pre-cleared by incubating with Protein A-Sepharose beads for 30 min at 4 °C. Protein A-Sepharose beads for immunoprecipitation were washed with lysis buffer at 2000 × g for 2 min. Proteins were then incubated for 2 h at 4 °C with Protein A-Sepharose beads and Neurologin-3 antibody (#129311, Synaptic Systems, 1:1000). Unbound proteins were removed by three washes with lysis buffer. Peptides were then eluted in lithium dodecyl sulfate buffer (106 mM Tris–HCl, 141 mM Tris-base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM Brilliant Blue, 0.175 mM Phenol Red, 10 mM DTT) at 70 °C for 10 min. Samples were frozen at −20 °C for storage.

Mass-spectrometry analysis

Mass-spectrometry analysis was performed by the University of Bristol Proteomics Facility. Each sample was separated as a lane on an SDS–PAGE gel and electrophoresis was performed until the samples had run ~3 cm...
into the separating gel. The gel lane was then cut into three slices and each slice subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd.). The resulting peptides were fractionated using an Ultimate 3000 nano-LC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (vol/vol) formic acid was injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250 μm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using seven gradient segments (1–6% solvent B over 1 min, 6–15% solvent B over 58 min, 15–32% solvent B over 58 min, 32–40% solvent B over 5 min, 40–90% solvent B over 1 min, held at 90% solvent B for 6 min and then reduced to 1% solvent B over 1 min) with a flow rate of 300 μl min⁻¹. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.1 kV using a stainless-steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 250 °C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyze the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300–2000 and the top 20 multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalized collision energy, 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt Mouse database (78740 sequences) using the SEQUEST algorithm. Peptide and searched against the UniProt Mouse database enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5%.

**Social interest**

Social odors originated from two cages of three C57Bl/6 male mice with the different parental origins, maintained for 6 days with the same home cage bedding to allow for a concentration of odorants. Before the test, swabs were wiped in a zig-zag pattern across the bottom surface of the cage to collect the olfactory cues. Mice were acclimatized for 30 min to the presence of a cotton swab prior to testing. Mice were then exposed to a cotton swab without odor followed by a cotton swab with a social odor. During the 2 min exploration periods, the time spent sniffing the swab on the first exposure to each odor was recorded manually and blind to the genotype.

**Vocalization**

We recorded ultrasonic vocalization (USV) emitted by the male mice exposed to a female mouse in estrus (1, 2).

All male mice were first habituated for 3 min to the arena. Following the habituation, an unfamiliar female mouse in estrus was added to the same arena for 3 min. During this time the mice could interact with each other freely in the arena. The male vocalization behavior was recorded by a preamplifier (UltraSoundGate 416 H, Avisoft Bioacoustics) connected microphone (UltraSoundGate CM16, Avisoft Bioacoustics) placed above the arena. As previously described frequencies were recorded and analyzed for the total number of emitted calls and the total time spent calling using SASLabPro (Avisoft Bioacoustics).

**Marble burying**

Mice were in individual cages (28 cm × 17 cm) each containing 20 marbles arranged in five rows of four marbles on top of 4 cm deep bedding over the time course of 30 min. The testing room used was dimly lit, with equal light distribution for all mice in the trial. A marble was buried when ≤50% of the marble was not visible. For counting and quantification, experimenters were blind to the genotype.

**Rotarod behavior**

Prior to the experiment mice were handled over the course of 2 days. During a trial, the rod accelerated to 40 rpm within 5 min. The latency to fall was evaluated across seven subsequent trials with a rest period of 5 min in between. The latency to fall was determined based on the time spent on the rod until the test mouse fell off, gripped to the rod and followed the rod for a full rotation or the testing trial ended after 5 min.

**Activity and anxiety behavior**

Spontaneous activity of the mice was tested in an open field arena (40 cm × 40 cm) for 20 min, in the dark. The mice were able to freely move and explore the environment during the test. The behavior was recorded by a computer linked video camera located above the arena. The arena was illuminated from the bottom by an infrared light source, for detection of the mice. EthoVision XT
was calculated as the number of spines per 10 (from four mice) per condition analyzed. Spine density dendrites.

acquisition and analyzed blinded to the experimental public domain). All images were coded at the time of projections of maximum intensity in ImageJ (NIH, USA, reconstructed into two dimensions according to Z-stack imaging of maximum intensity in ImageJ (NIH, USA, public domain). All images were coded at the time of acquisition and analyzed blinded to the experimental condition. Spines were manually identified and counted on 60–150 µm long dendrites, with N = 24 dendrites (from four mice) per condition analyzed. Spine density was calculated as the number of spines per 10 µm of dendrites.

Structural plasticity imaging in vivo

Dendritic spine imaging was performed in awake and head fixed Thy-1 GFP-expressing Cyfip1WT and Cyfip1Het mice were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were post-fixed overnight, stored in OCT at −80 °C, then cut coronally into 50 µm sections on a cryostat (Leica Biosystems, Germany) and mounted on a series of glass slides. Confocal images were acquired on a Zeiss LSM700 upright confocal microscope (Carl Zeiss, Welwyn Garden City, UK), using a ×43 water immersion lens (NA = 1.3). At least 10 Z-stack images (2048 × 2048 pixels) spaced 0.5 µm apart were acquired in the primary motor and visual cortices of each animal, focusing on secondary branching dendrites from cells. Series stacks were reconstructed into two dimensions according to Z-stack projections of maximum intensity in ImageJ (NIH, USA, public domain). All images were coded at the time of acquisition and analyzed blinded to the experimental condition. Spines were manually identified and counted on 60–150 µm long dendrites, with N = 24 dendrites (from four mice) per condition analyzed. Spine density was calculated as the number of spines per 10 µm of dendrites.

Immunohistology

Thy-1 GFP-expressing Cyfip1WT and Cyfip1Het mice were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were post-fixed overnight, stored in OCT at −80 °C, then cut coronally into 50 µm sections on a cryostat (Leica Biosystems, Germany) and mounted on a series of glass slides. Confocal images were acquired on a Zeiss LSM700 upright confocal microscope (Carl Zeiss, Welwyn Garden City, UK), using a ×43 water immersion lens (NA = 1.3). At least 10 Z-stack images (2048 × 2048 pixels) spaced 0.5 µm apart were acquired in the primary motor and visual cortices of each animal, focusing on secondary branching dendrites from cells. Series stacks were reconstructed into two dimensions according to Z-stack projections of maximum intensity in ImageJ (NIH, USA, public domain). All images were coded at the time of acquisition and analyzed blinded to the experimental condition. Spines were manually identified and counted on 60–150 µm long dendrites, with N = 24 dendrites (from four mice) per condition analyzed. Spine density was calculated as the number of spines per 10 µm of dendrites.

Structural plasticity imaging in vivo

Dendritic spine imaging was performed in awake and head fixed Thy-1 GFP-expressing Cyfip1WT and Cyfip1Het adult male mice (from postnatal day 60) with implanted cranial windows. Aseptic surgical procedures were conducted based on previously described protocols. Approximately one hour prior to cranial window surgery, animals were administered with the antibiotic Baytril (5 mg/kg, s.c.) and the anti-inflammatory drugs carprofen (5 mg/kg, s.c.) and dexamethasone (0.15 mg/kg, i.m.). Anesthesia was induced then maintained using isoflurane at concentrations of 4%, then 1.5–2%, respectively. After animals were secured in a stereotaxic frame, the scalp and periosteum were removed from the dorsal surface of the skull, and a custom head plate was attached to the cranium using dental cement (Super Bond C&B), with an aperture approximately centered over right M1. A 3 mm circular craniotomy was next performed, centered over the forelimb area using stereotaxic coordinates 1.3 mm anterior to the bregma and 1.2 mm lateral from the midline. The craniotomy was then closed with a glass insert constructed from three layers of circular no. 1 thickness glass (1 × 5 mm, 2 × 3 mm diameter) bonded together with an optical adhesive (Norland Products; catalog no. 7106). Mice were imaged one-week post-surgery. Initial dendritic spine imaging was followed up after 2, 7, and 9 days. In vivo 2-photon imaging was performed using a resonant scanning microscope (Thorlabs, B-Scope) with a ×16 0.8NA objective with 3 mm working distance (Nikon). eGFP was excited at 980 nm using a Ti:sapphire laser (Coherent, Chameleon) with a maximum laser power at the sample of 20 mW. Z stacks were acquired at a frame rate of ~30 Hz, with 20 frames per depth, from 15 depths spaced by 2 µm. Imaged stretches of dendrite close to parallel to the imaging plane were selected based on an eGFP expression which was traced back to the soma located in layer 5 (Z: 400–500 µm from brain surface). Cortical surface vascular landmarks were used to locate the same stretches of dendrite between sessions. During two-photon imaging animals were free to run on a custom designed fixed axis cylindrical treadmill, and data collection was limited to stationary periods to avoid locomotion related brain movement. Imaging data were acquired using Scanimage 4.1 (HHMI Janelia). Imaging data were first corrected for brain motion using an automated rigid registration algorithm implemented in Matlab (MathWorks). The 20 frames from each depth were then averaged and a maximum intensity projection calculated over the z planes which encompassed the stretch of the dendrite of interest. Formed and eliminated spines were manually counted and normalized to 100 µm of dendrite (Thy1-EGFP-Cyfip1Het N = 36, from four mice; Thy1-EGFP-Cyfip1WT N = 40, from four mice).

Statistical analysis

Prior the cluster analysis, every protein detected in immunoprecipitates from VNO of Nlgn3KO mice (Supplementary Table 2) was filtered out of the ones immunoprecipitated from wild-type mice VNO (Supplementary Table 2) and Nlgn3KO PvalbCre striatum and cerebellum (Supplementary Table 1), because proteins present in Nlgn3KO are likely to be false positives. The list of remaining proteins can be found in Supplementary Table 3. A protein was considered detected when its score was >0, therefore obtaining a binarized dataset containing proteins detected (score of 1) and non-detected (score of 0) for each technical triplicate and each anatomical region. We used SPSS Statistics® software to perform hierarchical cluster analysis based on the binarized score and determined that three clusters described the dataset best. We then performed a two-step analysis with three clusters and obtained the results presented in Fig. 1. Supplementary Table 4 contains the data structure, type of test used, observed power and N for Figs. 3 and 4. Each test included enough animals to reach a power close to or above 0.8. We designed our tests groups to have mice from the different groups tested at the same time. We used SPSS Statistics® software to systematically test for normality using Levene’s test. No animals were removed from the analyses. Pairwise comparisons were analyzed by two-tailed Student’s t-test for normally distributed
datasets or two-tailed Mann–Whitney’s test for non-normally distributed datasets. All datasets used for two-way non-repeated and repeated measure ANOVAs were normally distributed and, when appropriate, followed by post-hoc Bonferroni’s test.

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Author details

1School of Biosciences, Cardiff University, Cardiff CF10 3AX Wales, UK
2Neuroscience and Mental Health Research Institute, School of Medicine, Cardiff University, Cardiff CF24 4HQ, UK. Faculty of Medicine and Health Sciences, Universitat Internacional de Catalunya, Barcelona, Spain

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary information

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