Haploinsufficiency of the schizophrenia risk gene *Cyfip1* causes abnormal postnatal hippocampal neurogenesis through a novel microglia dependent mechanism

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Abstract

Genetic and epidemiological evidence implicate the immune system in the pathogenesis of schizophrenia but mechanistic insights are lacking. Here we show that haploinsufficiency of *Cyfip1* a candidate risk gene for schizophrenia in the pathogenic 15q11.2(BP1-BP2) deletion impacts on microglia the resident immune cells of the central nervous system to cause abnormal neurogenesis in the postnatal hippocampus. We use mouse models to first demonstrate that haploinsufficiency of *Cyfip1* leads to increased numbers of adult born hippocampal neurons without altering proliferation. We next show that the increased numbers of new born neurons are due to reduced apoptosis of immature neurons. We then demonstrate that apoptosis of immature neurons is controlled by secreted factors from microglia. Finally we show that haploinsufficiency of *Cyfip1* leads to a cell autonomous failure of microglia induced neuronal apoptosis, resulting in sparing of immature neurons that would normally have been culled at this developmental check-point, with implications for maladaptive function. Our findings provide a novel mechanism linking a psychiatric risk gene with microglial dysfunction in the hippocampus, a brain area with strong evidence for involvement in psychopathology.

Introduction

Schizophrenia is a disabling psychiatric condition, with high heritability. Recent large scale molecular genetic studies have identified up to 160 common and rare independent loci that increase risk for falling ill with the disease[1,2]. Schizophrenia risk loci are enriched within synaptic proteins[3] and histone modifiers[4]. There is also emerging evidence implicating risk loci related to the innate immune system[4,5]. Evidence from epidemiological data[6,7] and findings showing increased inflammatory factors in the circulation[8] and in post-mortem brain[9] of schizophrenia patients further implicate altered immune function in the development of disease symptoms.

Mechanistic insights linking immune changes to risk for psychopathology are lacking. However, recent interest has focused on microglia, the resident macrophage-like immune cells of the central nervous system, with data suggesting possible alterations in microglial functioning in both diagnosed patients and healthy people at high risk of schizophrenia[10–13]. The causal mechanism(s) by which microglia might contribute to pathogenesis remain unclear. However, there is increasing evidence that, in addition to canonical immune functions, microglia can also modify neuronal networks through synaptic pruning during development[14] and adulthood[15] and that this microglial-mediated pruning is important for normal brain and behavioural functioning[16]. Additionally, microglia can modulate neuronal firing through direct cell-cell contacts[17], and have been shown to be capable of phagocytosing both developing[18] and adult[19] neurons.

There is also previous evidence linking postnatal neurogenesis to schizophrenia including changes in cell proliferation and expression of immature neuronal markers in the hippocampus[20–22]. Adult born hippocampal neurons, up to 700 of which are estimated to be born per day in humans[23], develop through a well-defined series of cell divisions and differentiations, starting from proliferative stem cells and intermediate progenitors, through migratory neuroblasts and immature neurons, before eventually forming mature granule cells[24]. In rodent models, adult born hippocampal neurons have been shown to be involved in a number of behavioural processes spanning pattern separation[25,26],

spatial navigation[27,28], memory turnover[29], and acquisition[30] and extinction[31] of fear memory. Schizophrenia patients show impairments in many of these psychological processes[32–35]. Furthermore, atypical antipsychotics increase neurogenesis[36] and a number of functionally diverse schizophrenia risk genes, including *DISC1*[37], *SREB2*/*GPR85*[38], *CACNA1C*[39,40] *DGCR8*[41], and *miR137*[42], modify adult hippocampal neurogenesis in rodent models.

Here we examine the process of postnatal hippocampal neurogenesis using a mouse model of Cyfip1 haploinsufficiency. *Cyfip1* is a schizophrenia risk factor through its presence in the 15q11.2(BP1-BP2) copy number deletion. Loss of one copy of this interval leads to substantially increased disease risk in both Western[43,44] and Han Chinese populations[45]. *CYFIP1* haploinsufficiency is likely a key contributor to the 15q11.2(BP1-BP2) psychiatric phenotype due to the established roles of *CYFIP1* in synaptic function. CYFIP1 has been shown to be involved in alterations in dendritic spine morphology and branching[46–48] and furthermore CYFIP1 protein interacts with fragile X mental retardation 1 (FMRP), the protein gene product of FMR1, to suppress translation of up to 800 target transcripts[49]. Alterations in FMR1, causative for Fragile X syndrome, are also associated with a range of psychiatric symptoms[50]. Additionally, CYFIP1 protein interacts with Wiskott-Aldrich syndrome protein family member 1 (WAVE1), a key mediator of cytoskeleton dynamics, to modulate ARP2/3 dependant actin branching[51].

In our experiments, we demonstrate a novel function of *Cyfip1* in the control of postnatal neurogenesis in the hippocampus. Specifically, we show that *Cyfip1* is highly expressed in microglia and is involved in inducing apoptosis of new born immature neuron in a process requiring microglial secreted factors. We also show that this process is gene-dose sensitive and that haploinsufficiency of *Cyfip1,* recapitulating a genetic risk factor for schizophrenia, impairs the ability of microglia to carry out this function leading ultimately to an excess of mature new born neurons within the dentate gyrus. These findings provide a novel mechanism linking a penetrant psychiatric risk gene candidate with microglial dysfunction in the hippocampus, a brain area central to disease pathology.

Materials and methods

For full materials and methods, see Supplementary Information.

Cyfip1 heterozygous knockout mice

Cyfip1$^{tm2a(EUCOMM)Wtsi}$ animals (MGI:5002986, referred to hereafter as *Cyfip1*$^{+/-}$)were maintained heterozygously on a C57/BL6J background. These animals have significant reductions at both mRNA and protein levels[46]. Animals used were of mixed sex and 8-12 weeks old. *Cyfip1*$^{+/-}$ and wildtype littermates were kept in conventional cages with 12h light-dark cycle and ad libitum access to water and food at all time.

Tissue harvesting and immunohistochemistry

Paraformaldehyde fixed brains from 8-12 week old *Cyfip1*$^{+/-}$ and wildtype littermates were prepared as previously described[52]. Free floating 40 µm cryostat sections were used for immunohistochemistry. Immunohistochemistry was performed as previously described[52], with a 1:12 stereotactic sampling rate. Detection of cleaved caspase 3 used a modified protocol, where primary antibodies were incubated for 48 h in blocking solution at 4°C. Primary and secondary antibodies are described in table S1.

Primary hippocampal progenitor culture

Hippocampal progenitors were isolated from P7-P8 animals. Isolated hippocampi were dissociated in papain and progenitors were enriched through Optiprep density gradient centrifugation. Cells were cultured on poly-L-lysine and laminin in the presence of EGF and FGF2.

Primary microglial culture

Microglia were isolated from P7-P8 whole brain mixed glia through the shake-off method.

Immunocytochemistry

Fixed cultures were blocked and permeabilized with 5% donkey serum and 0.1% Triton X100 in PBS for 30 minutes at room temperature. Primary antibodies were applied in the same solution overnight at 4 °C. Antibodies are detailed in Table S1. Following three washes in PBST, relevant secondary antibodies were applied in PBST for two hours at room temperature. For identification of microglia, Alexa 568 conjugated IB4 (Thermo Fisher I21412) was added to secondary antibody solutions at 2.5 µg/ml. Cells were washed, counterstained with DAPI, and mounted for microscopy.

Conditioned medium

Microglia conditioned medium was prepared by incubating $5x10^5$ microglia per ml in progenitor medium for 24 h. 25% conditioned medium was added to hippocampal cultures.

Secretomic analysis

For further characterisation of microglial phenotype, comprehensive secretomic analysis was performed using a Luminex™ multiplex bead array (R&D systems, LXSAMSM) on culture supernatant, according to manufacturer's protocol. Full list of analytes is in Table S2

Results

Cyfip1$^{+/-}$ animals have normal proliferation but increased numbers of adult born immature neurons in the hippocampus

We modelled *CYFIP1* haploinsufficiency using an established heterozygous *Cyfip1*$^{+/-}$ knock out mouse line[46] (see Methods and Supplementary Information for details) We first investigated cell proliferation in the dentate gyrus of the hippocampus in brain sections. Mixed sex *Cyfip1*$^{+/-}$ animals and littermate controls received a single injection (i.p) of 100 mg/kg BrdU 6 hours prior to sacrifice. No significant group differences in cells positive for BrdU (197.3±11.1 vs 182.9±11.7 cells/mm^2, $t_{(7)}$=0.86, p=0.41) or the cell cycle marker Ki67 (422.4±24.9 vs 371.8±30.8 cells/mm^2, $t_{(7)}$=1.27, p=0.25) were found (fig 1a-d), indicating no effects on cell proliferation. However, staining for the early neuronal marker doublecortin (DCX) revealed a significant increase of immunoreactive cells in the *Cyfip1*$^{+/-}$ animals (367.0±21.9 vs 550.6±39.4 cells/mm^2, $t_{(15)}$=4.38, p=6.21x10^{-4}, fig 1e-g), consistent with the presence of increased numbers of immature neurons. In a 30 day BrdU pulse-chase protocol we observed increased numbers of mature post-mitotic BrdU+/NeuN+ labelled cells in the dentate gyrus of *Cyfip1*$^{+/-}$ animals (42.9±7.6 vs 61.5±3.9 cells/mm^2, $t_{(10)}$=4.54. p=6.8x10^{-4}), as well as an increased fraction of NeuN+ cells in the total BrdU+ population (51.0±2.7% vs 60.5±0.8%, $t_{(10)}$=3.95, p=0.002) (fig 1h-l). These data indicated that the excess numbers of immature neurons seen in the *Cyfip1*$^{+/-}$ animals were maintained into maturation. We found no significant effects of sex of the animal (see fig S1).

Increased neuronal numbers in *Cyfip1* haploinsufficiency is due to decreased apoptosis

To investigate the cell-intrinsic effects of *Cyfip1* haploinsufficiency independent of niche effects we used primary hippocampal progenitor cultures. The *in vitro* data corroborated the *ex vivo* finding, showing an increase in the numbers of DCX immunoreactive cells (as fraction of total cell number) in the cultures prepared from *Cyfip1*$^{+/-}$ animals (5.5±0.5% vs 8.8±1.4%, $t_{(16)}$=2.78, p=0.014, fig 2a,b) in the absence of any effects on proliferation (fig S2). The primary cultures showed no group differences in the proportions of earlier progenitors of the 1/2a GFAP+/nestin+ type (9.4±1.2% vs 10.0±1.7%,

$t_{(35)}$=0.76, p=0.79), or 2b GFAP-/nestin+/DCX- types (22.1±1.6% vs 20.2±2.5%, $t_{(35)}$=0.67, p=0.51), (fig 2c-e), indicating no effects on the earliest stages of neurogenesis. We next assessed general cell viability and survival. We saw a significant increase in the proportion of viable MitoTracker+ cells (81.2±1.7% vs 86.7±2.9%, $t_{(13)}$=3.85, p=0.002) and fewer propidium iodine positive dead/dying cells (8.4±0.8% vs 5.4±0.5%, $t_{(14)}$=2.63, p=0.019)(fig 2f-h). We then investigated apoptosis specifically in nestin+ and DCX+ populations. We found a marked reduction in the fraction of DCX+ /nestin- cells positive for cleaved-caspase-3 (25.9±5.5% vs 7.2±2.5%, $t_{(16)}$=2.32, p=0.034, fig 2i,j). As expected, we detected negligible levels of cleaved-caspase-3 immunoreactivity in nestin-positive cells. Together, these data suggested Cyfip1 haploinsufficiency impaired a normal homeostatic mechanism for modulating newborn neuron number, leading to a greater number surviving to go on to maturity. To confirm the effects on cell death due to apoptosis were not an artefact of the culture system we also showed that there were significantly fewer cleaved-caspase-3 immunoreactive cells in the dentate gyrus of *Cyfip1*$^{+/-}$ animals (2.1±0.2 vs 1.2±0.1 cells/mm^2, $t_{(23)}$=3.01, p=0.006, fig 2k,l), again demonstrating a high level of concordance between effects in cell culture and intact brain tissue.

Apoptosis of newly born neurons is mediated by microglia secreted factors

Given the known roles microglia play in earlier stages of neurogenesis[53], we speculated that microglia may play a role in the survival and death of adult new born immature neurons. To test this we first depleted microglia from wildtype primary hippocampal progenitor cultures using the specific microglia toxin Mac-1-SAP, targeting CD11b expressing cells. We established the presence of microglia in the culture system (fig S3) and the efficacy of the toxin against primary mouse microglia (fig 3a). We then investigated cell type specific apoptosis. Depletion of microglia led to an increase in the proportion of DCX+ cells (7.5±1.6% vs 12.7±0.3%, $t_{()}$=3.19, p=0.019, fig 3b), and a specific decrease in the fraction of apoptotic DCX+ cells (13.1±3.1% vs 2.7±1.7%, $t_{(7)}$=2.93, p=0.026, fig 3c), without any associated changes in the nestin+ population (50.7±2.7 %vs 53.8±1.0%, $t_{(7)}$=1.07, p=0.32, fig 3d).

We then assessed the effects of additional exogenous microglia on a permeable membrane insert (fig 3e) allowing the diffusion of soluble factors, but no direct cell-cell contact. Under these conditions we observed the opposite effects to when we depleted microglia, that is, a decreased proportion of DCX+ cells (5.9±0.8% vs 1.9±0.5%, $t_{(15)}$=4.36, p=6.5x10^{-4}, fig 3f), increased apoptosis in the DCX+ population (14.8±4.7% vs 51.7±9.0%, $t_{(15)}$=3.27, p=0.006, fig 3g) and no effect on the nestin+ population (58.4±3.3% vs 62.7±3.0%, $t_{(15)}$=0.98, p=0.34, fig 3h). These experiments provide converging evidence for a previously unknown function of microglia in inducing apoptosis of immature neurons in hippocampus mediated, at least in part, by soluble factors.

Haploinsufficiency of *Cyfip1* inhibits the ability of microglia to support apoptosis of new born immature hippocampal neurons.

Having established a novel microglial dependent mechanism for regulation of neuronal survival we next tested whether effects on this process could underlie the neurogenesis effects of *Cyfip1* haploinsufficiency. First we established there was no genotype difference in the density of $Iba1^+$ microglia in hippocampus ex vivo (177.1±4.1 vs 162.9±8.7, $t_{(12)}$=1.22, p=0.25, fig 4a,b). We next showed that *Cyfip1* is highly expressed in primary wildtype microglia (comparable to the levels of the housekeeping gene GAPDH) and expression is strongly up-regulated (15.2±0.95-fold) following activation with lipopolysaccharide (LPS), ($t_{(5)}$=14.8, p=0.001, fig 4c) suggesting Cyfip1 is involved in microglial regulation. Then we compared LPS-activation of wildtype and *Cyfip1*$^{+/-}$ primary microglia indexed by CD68 immunoreactivity. Both cultures showed evidence of a robust activation with a significant treatment effect ($F_{(1,48)}$=9.86, p=0.002). There was also a significant genotype effect ($F_{(1,48)}$=4.27, p=0.043) with no interaction ($F_{(1,48)}$=0.93, p=0.33) reflecting a degree of blunting of activation in *Cyfip1*$^{+/-}$ cells. (fig 4d).

Given the likely role of microglial secreted factors, and to further characterise the effects of Cyfip1 haploinsufficiency on microglia, we analysed secreted factors from wildtype *Cyfip1*$^{+/-}$ cultures using the Luminex™ multiplex bead array. As shown in fig 3e, we found detectable levels of TNFα, IL6, IL10,

CCL4, CCL5, CCL7, CCL12, CXCL1, CXCL2, CXCL10, Serpin E1 and VEGF. All but CXCL2 and Serpin E1 were significantly up-regulated following LPS stimulation. However, no significant effects of genotype were found with respect to any of the molecules we were able to assay (full list of analytes in Table S2 and full statistics in Table S3).

The range of molecules in the Luminex™ assay is limited so we reasoned that other soluble factors from microglia may have been involved in the altered apoptosis associated with *Cyfip1* haploinsufficiency. We addressed this directly in an unbiased way using a cross-genotype conditioned medium approach where medium obtained from wildtype and *Cyfip1*$^{+/-}$ microglia preparations was added to both the wildtype and *Cyfip1*$^{+/-}$ hippocampal progenitor enriched cultures (see fig 5A&E for schematic of design) and measured cell proportions and levels of apoptosis. In the wildtype progenitor cultures adding conditioned medium from wildtype microglia resulted in a significant reduction in the proportion of DCX+ cells, compared to unstimulated cells, whereas *Cyfip1*$^{+/-}$ conditioned medium had no effect (5.4±0.9% vs 2.9±0.2% vs 5.4±0.3% for unstimulated, wildtype stimulated and Cyfip1$^{+/-}$ stimulated respectively, main effects of genotype $F_{(2,9)}$=6.31, p=0.019, fig 5b). *Post hoc* testing confirmed significant differences between controls and wildtype medium (p=0.031) and between those stimulated with wildtype and *Cyfip1*$^{+/-}$ medium (p=0.033). This pattern of effects was mirrored inversely in the fraction of DCX+ cells that were also immunopositive for cleaved-caspase- 3, with only conditioned medium from wildtype microglia cultures increasing apoptosis (16.1±3.6% vs 34.5±3.5% vs 14.8±2.5%, main effects of genotype $F_{(2,9)}$=11.57, p=0.003, fig 5c). Again, *post hoc* testing showed the wildtype stimulated condition was significantly different from controls and *Cyfip1*$^{+/-}$ stimulated condition (p=0.007 and p=0.005, respectively). Consistent with the conclusion that these effects were limited to the DCX+ immature neuron population of cells, the proportion of cultures that was nestin+ was unchanged across all conditions (65.0±5.2% vs 60.7±4.4% vs 57.1±4.1%, $F_{(2,9)}$=0.75, p=0.50, fig 5d).

Importantly, identical patterns of effects were seen in the *Cyfip1*$^{+/-}$ hippocampal progenitor cultures. Using an identical experimental design (fig 5d), we found that conditioned medium from wildtype

microglia, but not from *Cyfip1*$^{+/-}$ microglia, decreased the proportion of DCX+ cells (8.1±0.6% vs 5.1±0.2% vs 9.0±0.7%, main effects of genotype $F_{(2,9)}$=13.82, p=0.002, fig 5e) with post hoc analysis confirming wildtype microglia medium stimulated conditions were significantly different from control and *Cyfip1*$^{+/-}$ stimulated conditions (p=0.008 and p=0.002, respectively). Similarly, only conditioned medium from wildtype microglia was associated with a significant increase in the proportion of apoptotic DCX+ cells (12.8±2.3% vs 28.3±5.0% vs 13.0±0.9%, main effects of genotype $F_{(2,9)}$=6.12, p=0.021, fig 5f) with post hoc analysis confirming the wildtype stimulated condition was significantly different from the unconditioned medium controls and *Cyfip1*$^{+/-}$ stimulated condition (p=0.007 and p=0.005, respectively). As before, no effect of any of the medium conditions was seen on the nestin-positive population (64.7±2.6% vs 64.0±2.3% vs 57.5±3.3%, $F_{(2,9)}$=2.13, p=0.18, fig 5f). These data provide converging evidence for induction of neuronal apoptosis through factors secreted by microglia, a process which is disrupted in *Cyfip1*$^{+/-}$ animals.

Discussion

The immune system has been implicated in risk for schizophrenia[6–9]. Here we have demonstrated a previously unknown link between microglia, the resident immune cells in the brain, and haploinsufficiency of a genetic risk candidate for schizophrenia, *Cyfip1*. We report that *Cyfip1*$^{+/-}$ animals have larger numbers of surviving newly born neurons in hippocampus, in the absence of increases in numbers of stem cells or rate of proliferation, and provide evidence that this is the result of a specific failure in microglia-induced neuronal apoptosis (see summary of proposed mechanism in fig 5i). Our data are consistent with a novel role for microglia in controlling adult hippocampal neurogenesis, a cellular process with a suggested role in the symptomatology of schizophrenia and other psychiatric diseases[54].

Despite recent debate regarding the extent to which adult hippocampal neurogenesis occurs in humans[55], revolving largely around the use of different cellular markers, the location, cell types and numbers involved in this process have now been well characterised though several different means[23,56]. Although we have used P7-P8 animals to generate sufficient primary cells, by this stage the niche organisation and marker expression patterns are comparable to adults[57]. The large degree of cross-over between in vivo and in vitro approaches strongly suggests the data obtained in the culture systems are applicable to in vivo adult neurogenesis.

Our study is the first to demonstrate a direct effect of microglial soluble factors on the survival of immature neurons as a homeostatic mechanism. In contrast to earlier findings examining the effect of microglia on adult neurogenesis[58–60], we show this occurs in the absence of overt inflammatory disease or microglial activation. Nonetheless, our data indicate this is a mechanism susceptible to pathogenic changes as evidenced by the effects of *Cyfip1* haploinsufficiency on the ability of microglia to regulate apoptosis of immature neurons. Importantly, the inability of *Cyfip1*$^{+/-}$ microglia to induce neuronal apoptosis seems to be a specific deficit, rather than as a result of generalised microglial dysfunction. We showed a mild activation deficit as measured by CD68 levels in *Cyfip1*$^{+/-}$ microglia. In

our case however, overall secretion of cytokines and chemokines, both at baseline and following activation, was unaffected. This suggests that, whilst *Cyfip1* is strongly up-regulated following activation, and it may play some role during the response to inflammatory stimuli, it is not crucial for a robust immune response in microglia.

The failure of neurons to enter microglia-induced apoptosis under conditions of *Cyfip1* haploinsufficiency could be due to a failure of the microglia to induce the apoptosis, or a failure of neurons to respond appropriately, or both. However, the fact that conditioned medium from wildtype microglia was able to induce apoptosis in *Cyfip1*$^{+/-}$ microglia demonstrates unequivocally that these neurons are capable of responding to soluble pro-apoptotic signals, and the primary deficit is in the microglia, not the neurons. Moreover, the inability of *Cyfip1*$^{+/-}$ microglia conditioned medium to induce apoptosis further confirmed the deficit is in the microglia not the neurons. There is also the possibility of the *Cyfip1*$^{+/-}$ microglia secreting higher levels of anti-apoptotic factors, rather than failing to secrete pro-apoptotic factors. However, this does not fit our data as were this to be the case we would expect a decrease in apoptosis in wild-type cultures exposed to conditioned medium from *Cyfip1*$^{+/-}$ microglia, which we did not observe.

We have demonstrated that soluble factors from microglia are involved in controlling numbers of immature neurons, and that haploinsufficiency of *Cyfip1* leads to reduced apoptosis via an effect on factors secreted from microglia, however the soluble factor(s) responsible remain to be identified. *Cyfip1* has numerous pleotropic effects and it is known that the types of actin remodelling *Cyfip1* can influence is involved in cytokine secretion[61]. However, no genotype difference in secreted levels of a number of common cytokines and chemokines was apparent and further work is needed to identify the soluble factor(s) involved in the altered molecular signalling between microglia and developing new born neurons.

The effects of *Cyfip1* haploinsufficiency were highly cell-type specific and appeared limited to the stage of cell maturity where neuronal progenitors are fully committed to a neuronal phenotype, a stage of

development characterised by high levels of apoptosis (see figure 5i). However, the increase in surviving neurons we observed persisted for up to at least 30 days post-division, at which point, given the expression of the mature neuronal marker NeuN, it is highly likely these cells have integrated into the local circuitry. Indeed, it is known that at 30 days of maturity, newly born neurons are already connected to the local granule cell population[62]. Consequently, it is possible that functional effects on brain and behaviour could be exerted at several points along the developmental pathway from the immature neuron stage to the fully integrated mature granule cell in the dentate gyrus. Studies which are able to track the electrophysiological and other functional properties of *Cyfip1*$^{+/-}$ adult born neurons as they differentiate, migrate, and form synaptic contacts would be required to answer this question.

It is becoming increasingly evident that microglia play multiple roles in brain function beyond their involvement in classical immune homeostasis. Our findings identify a novel mechanism by which microglia are involved in the regulation of hippocampal neurogenesis in a process that is disrupted by *Cyfip1* haploinsufficiency, leading to an excess of adult born neurons. The 15q11.2(BP1-BP2) deletion is not solely associated with schizophrenia, it also markedly increases risk for autism spectrum disorders[63], ADHD and developmental delay[64], and epilepsy[65]. Whether altered microglia functioning due to *Cyfip1* haploinsufficiency, impacting on adult hippocampal neurogenesis, also plays a part in these disorders is an open question, but all have been associated with alterations in immune system and microglia functioning[66,67]. Moreover, as noted previously, a number of schizophrenia risk genes, including *DISC1*[37], *SREB2/GPR85*[38], *CACNA1C*[39,40] *DGCR8*[41], and *miR137*[42], have also been shown to modify adult hippocampal neurogenesis in model systems. Whilst the effects are distinct from those of *Cyfip1*, each risk gene impacting on neurogenesis in its own way, this convergence of function may be indicative of a common pathogenic route for a number of diverse psychiatric risk genes.

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Contributions

NH and LSW designed the study. NH designed and performed experiments, and drafted the manuscript. JC performed animal husbandry/genotyping. LJW optimised and assisted with primary culture. WPG and LSW initiated the study and assisted with data interpretation. MJO, WPG, JH, and LSW critically appraised the manuscript. All authors approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1: *Cyfip1 haploinsufficiency increases neurogenesis in vivo in the absence of changes in proliferation.* a) *Experimental scheme*, proliferation rates were studied with a 6h pulse of BrdU. b, c) Representative immunohistochemistry showing distribution and location of proliferative (Ki67+) and dividing (Ki67+BrdU+) cells in wild-type and *Cyfip1*$^{+/-}$ animals (n=4 each), respectively. d) Quantification showed no difference in the total numbers of proliferative cells or number of cell division during the 6 hour experiment. e, f) Representative staining for DCX in wild-type and *Cyfip1*$^{+/-}$ animals (n=8 each), respectively. g) Quantification showed a significantly larger number of immature neurons in the *Cyfip1*$^{+/-}$ animals. h) *Experimental scheme*, cell maturation was studied with a 30 day BrdU pulse-chase. i,j) Representative images showing number and localisation of BrdU+ and NeuN+ cells. Arrowheads indicate BrdU+ cells k) *Cyfp1*$^{+/-}$ animals (n=6) had significantly higher numbers of BrdU+NeuN+ cells than the wild-type animals (n=5). l) The fraction of BrdU+ cells which had matured into NeuN+ cells was significantly increased in the *Cyfip1*$^{+/-}$ animals. All data are shown as mean±SEM.

Figure 2: *Primary hippocampal progenitor cultures prepared from P7-P8 brain confirm ex vivo findings and indicate that increased neuronal numbers in Cyfip1*$^{+/-}$ *animals is due to reduced apoptosis.* a) Representative staining for immature neuron marker DCX and hippocampal marker Prox1 confirm cultures contain hippocampus-derived developing neurons. b) Cyfip1$^{+/-}$ animals showed a significantly higher proportion of DCX+ cells in the total DAPI+ population (n=17). c) Representative staining for progenitor markers GFAP and nestin showed cultures contained a mixed population of progenitors at differing stages of development. d) There was no significant genotype difference in the proportion of early GFAP+/nestin+ cells (n=36). e) Again, there was no significant genotype difference in the proportion of later GFAP-/nestin+ cells (n=36). f. Live imaging showed cultures are comprised of healthy, MitoTracker+ cells, with some dying PI+ cells. g) A small but significant increase in the proportion of MitoTracker+ cells was observed in the *Cyfip1*$^{+/-}$ cultures. (n=15) h) Conversely, a significant decrease in the proportion of PI+ cells was seen in the *Cyfip1*$^{+/-}$ cultures (n=14). i) Representative staining for the later apoptotic marker cleaved caspase with DCX and nestin showed

specific neuronal apoptosis. Arrowhead indicates an apoptotic neuron. j) The fraction of neurons undergoing apoptosis was significantly lower in the *Cyfip1*$^{+/-}$ cultures (n=17). l) Representative image of apoptotic cells in the detate gyrus. L) As seen *in vitro*, *Cyfip1*$^{+/-}$ animals (n=13) have significantly fewer apoptotic cells than wild type animals (n=12). All data are shown as mean±SEM.

Figure 3: *Microglia regulate number and apoptosis rates of immature neurons.* a) *Experimental setup*, Mac-1-Sap was present in wildtype hippocampal progenitor cultures from 2 hours after isolation until 3 days *in vitro*. Dose-response curves of microglial death after Mac-1-SAP administration show near-complete depletion of Iba1+ microglia was achieved within 48 h (n=6) b). Cultures where microglia were depleted (n=4) showed a higher proportion of DCX+ cells than controls (n=4). c) Correspondingly, the fraction of DCX cells undergoing apoptosis, as marked by cleaved caspase 3 staining, was significantly decreased in the absence of microglia. d) The proportion of nestin+ cells was unaffected by microglia depletion. e) *Experimental setup*, Membrane inserts containing wild-type microglia prepared from P7-8 brain were added to cultures 2 hours after progenitor isolation and were present throughout the remainder of the experiment. Due to the experimental conditions, no cell-cell contact between microglia and progenitors was possible, but secreted factors were free to diffuse. f) The presence of microglia-containing inserts (n=8) significantly decreased the proportion of DCX expressing cells compared to controls (n=8). g) Conversely, the added microglia significantly increased apoptosis in DCX+ cells. H) No effect was seen in nestin expressing cells. All data are shown as mean±SEM.

Figure 4: *Microglia alterations due to Cyfip1 haploinsufficiency.* a) Representative staining for Iba1 shows the distribution of microglia in the dentate gyrus. b) Quantification of microglial numbers showed no significant genotype differences (WT n=5, *Cyfip1*$^{+/-}$ n=8. c) Quantitative PCR for *Cyfip1* transcript in wildtype primary microglia showed a highly significant upregulation following activation

by 10 µg/ml LPS (n=3 each). d) Cyfip1$^{+/-}$ primary microglia showed a significant activation deficit, as measured by CD68 levels. e) No significant effects of genotype in levels of microglia secreted factors in culture medium assayed using the Luminex™ system at baseline and following activation with LPS (n=10 for all conditions). All data are shown as mean±SEM.

Figure 5: *Cyfip1$^{+/-}$ microglia are unable to induce neuronal apoptosis.* a) *Experimental design*, Wild-type hippocampal progenitors were exposed to conditioned medium from either wildtype or *Cyfip1$^{+/-}$* whole brain microglia. b) In wildtype cultures, conditioned medium from wildtype microglia induced a significant reduction in the proportion of DCX+ cells, whereas conditioned medium from *Cyfip1$^{+/-}$* microglia was unable to do this (n=4 in all conditions). c) A similar but inverse pattern was seen in neuronal apoptosis where wildtype microglia medium increased apoptosis in DCX+ immature neurons but *Cyfip1$^{+/-}$* microglia medium did not. d) Nestin+ cells were not significantly affected by the presence of microglial factors. e) *Experimental design*, *Cyfip1$^{+/-}$* hippocampal progenitors were exposed to conditioned medium from either wildtype or *Cyfip1$^{+/-}$* microglia. f) As seen in the wildtype cultures, a significant decrease in DCX+ cells was observed only after stimulation with medium from wildtype microglia (n=4 in all conditions). g) Similarly, medium from *Cyfip1$^{+/-}$* microglia was unable to induce apoptosis in *Cyfip1$^{+/-}$* progenitors. h) Again, the nestin^{+} population was unaffected. All data are shown as mean±SEM. i) Model for effects of Cyfip1 haploinsufficiency. Progression of cells through the different stages of neurogenesis is unaltered though the early radial glial and intermediate progenitor stages, with no changes in number of proliferation. However, when the doublecortin positive stage of development is reached, there is a failure of *Cyfip1$^{+/-}$* microglia to induce neuronal apoptosis, as takes place in wild-type animals. This leads to the survival of a significantly larger number of immature neurons into maturity.

Figure S1: *In vivo* neurogenesis phenotype split by sex. A) Analysis of proliferation indexed by a 6 h BrdU pulse with Ki67 immunohistochemistry show no effects of sex or genotype. B) Immunohistochemistry for the immature neuronal marker DCX show a significant effect of genotype in the absence of any effects of sex. C) Analysis of mature adult born neurons, by NeuN immunohistochemistry 30 days after BrdU pulsing, showed a significant effect of genotype, again in the absence of any sex effects.

Figure S2: Proliferation in primary hippocampal cultures. Following withdrawal of growth factors, cells were given a 6 h EdU pulse. As seen following the *in vivo* BrdU pulse experiments, there are no differences in the total number of Ki67+ cells in the cell cycle or the fraction that had undergone cell division (EdU+).

Figure S3 – Primary cultures contain Iba1+ microglia

Figure S4 – Representative genotyping image for the *Cyfip1*$^{+/-}$ mouse line.

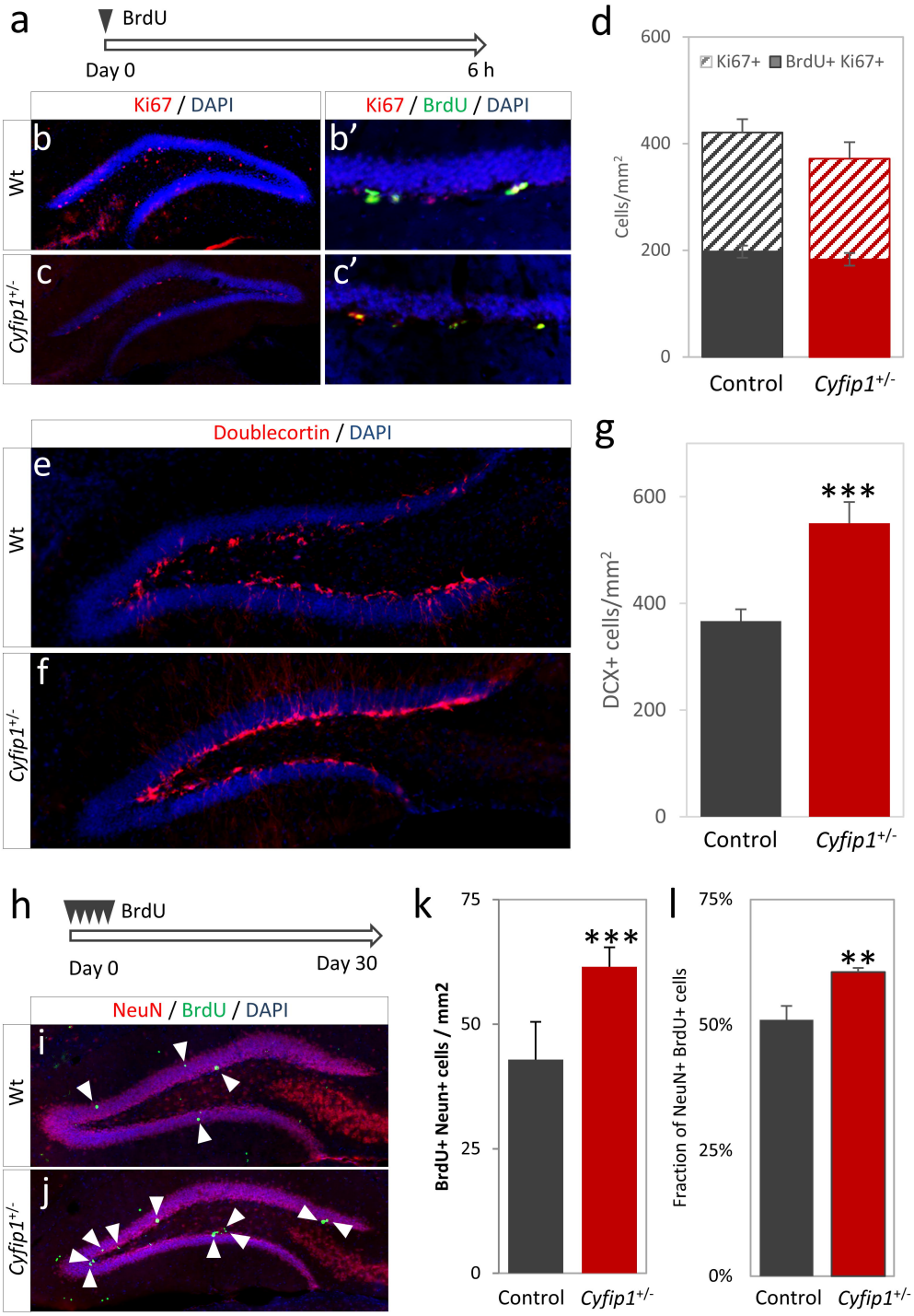

a
BrdU
Day 0
6 h
Ki67 / DAPI
Ki67 / BrdU / DAPI
Wt
Cyfip1+/-
b
b'
c
c'
d
Ki67+
BrdU+ Ki67+
Cells/mm2
600
400
200
0
Control
Cyfip1+/-
Doublecortin / DAPI
e
f
g
DCX+ cells/mm2
***
h
BrdU
Day 0
Day 30
NeuN / BrdU / DAPI
i
j
k
BrdU+ Neun+ cells / mm2
75
50
25
0
l
Fraction of NeuN+ BrdU+ cells
75%
50%
25%
0%
**

Figure 1

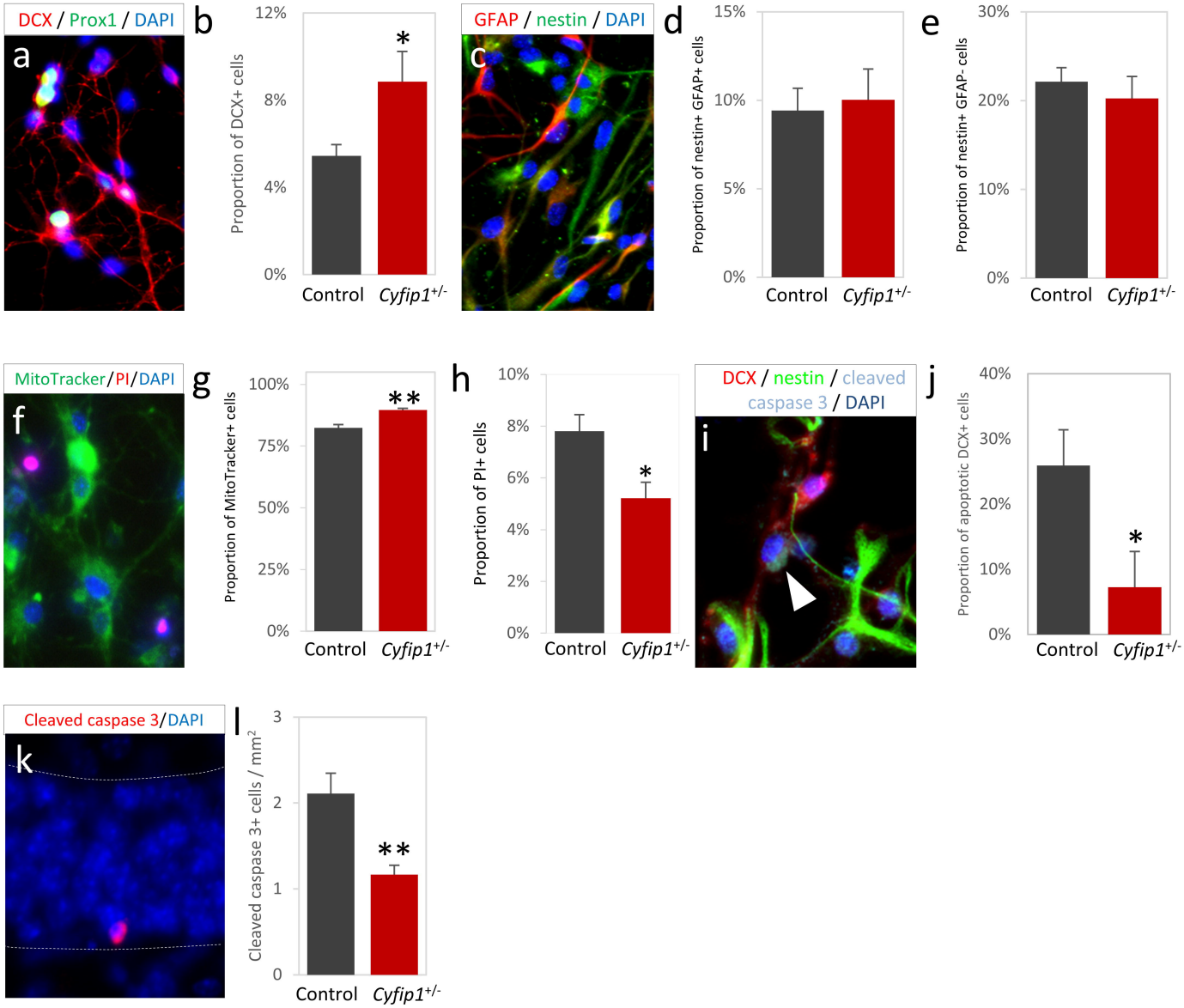

DCX / Prox1 / DAPI
a
b
12%
8%
4%
0%
Proportion of DCX+ cells
*
Control
Cyfip1+/-
GFAP / nestin / DAPI
c
d
15%
10%
5%
0%
Proportion of nestin+ GFAP+ cells
Control
Cyfip1+/-
e
30%
20%
10%
0%
Proportion of nestin+ GFAP- cells
Control
Cyfip1+/-
MitoTracker/PI/DAPI
f
g
100%
75%
50%
25%
0%
Proportion of MitoTracker+ cells
**
Control
Cyfip1+/-
h
10%
8%
6%
4%
2%
0%
Proportion of PI+ cells
*
Control
Cyfip1+/-
DCX / nestin / cleaved caspase 3 / DAPI
i
j
40%
30%
20%
10%
0%
Proportion of apoptotic DCX+ cells
*
Control
Cyfip1+/-
Cleaved caspase 3/DAPI
k
l
3
2
1
0
Cleaved caspase 3+ cells / mm2
**
Control
Cyfip1+/-

Figure 2

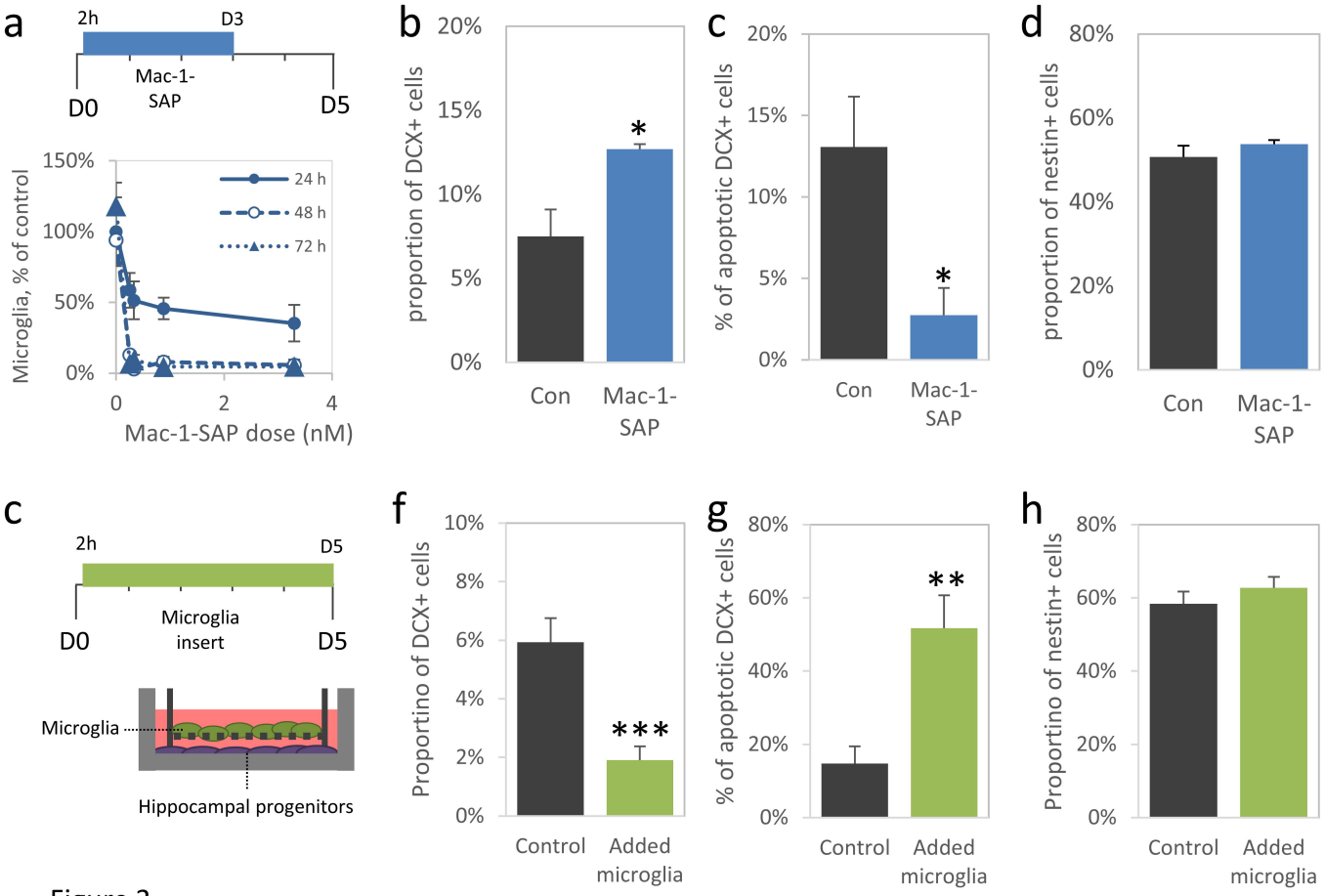

a
2h
D3
Mac-1-SAP
D0
D5
Microglia, % of control
150%
100%
50%
0%
24 h
48 h
72 h
0
2
4
Mac-1-SAP dose (nM)
b
proportion of DCX+ cells
20%
15%
10%
5%
0%
*
Con
Mac-1-SAP
c
% of apoptotic DCX+ cells
20%
15%
10%
5%
0%
*
Con
Mac-1-SAP
d
proportion of nestin+ cells
80%
60%
40%
20%
0%
Con
Mac-1-SAP
c
2h
D5
Microglia insert
D0
D5
Microglia
Hippocampal progenitors
f
Proportino of DCX+ cells
10%
8%
6%
4%
2%
0%
***
Control
Added microglia
g
% of apoptotic DCX+ cells
80%
60%
40%
20%
0%
**
Control
Added microglia
h
Proportino of nestin+ cells
80%
60%
40%
20%
0%
Control
Added microglia

Figure 3

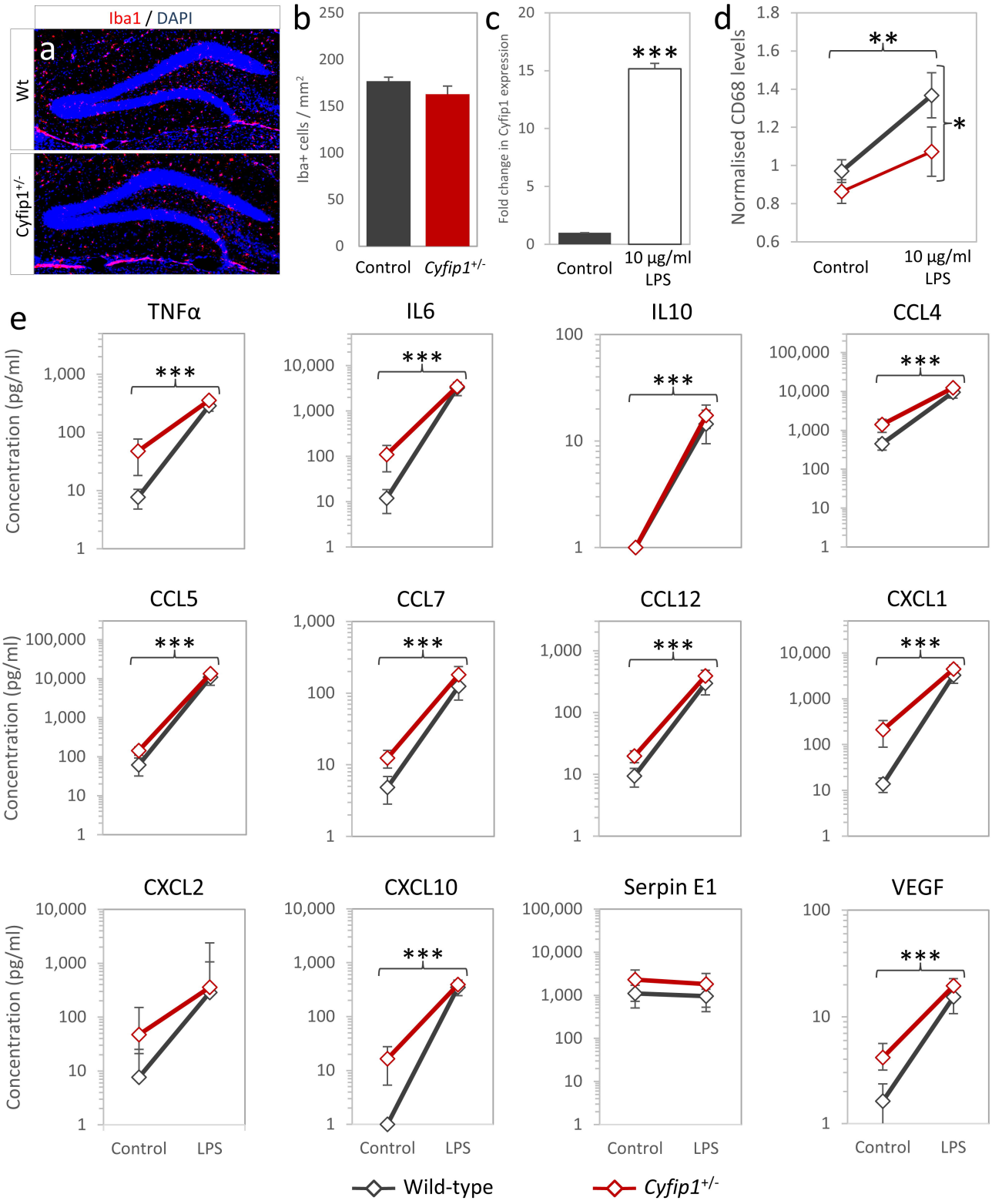
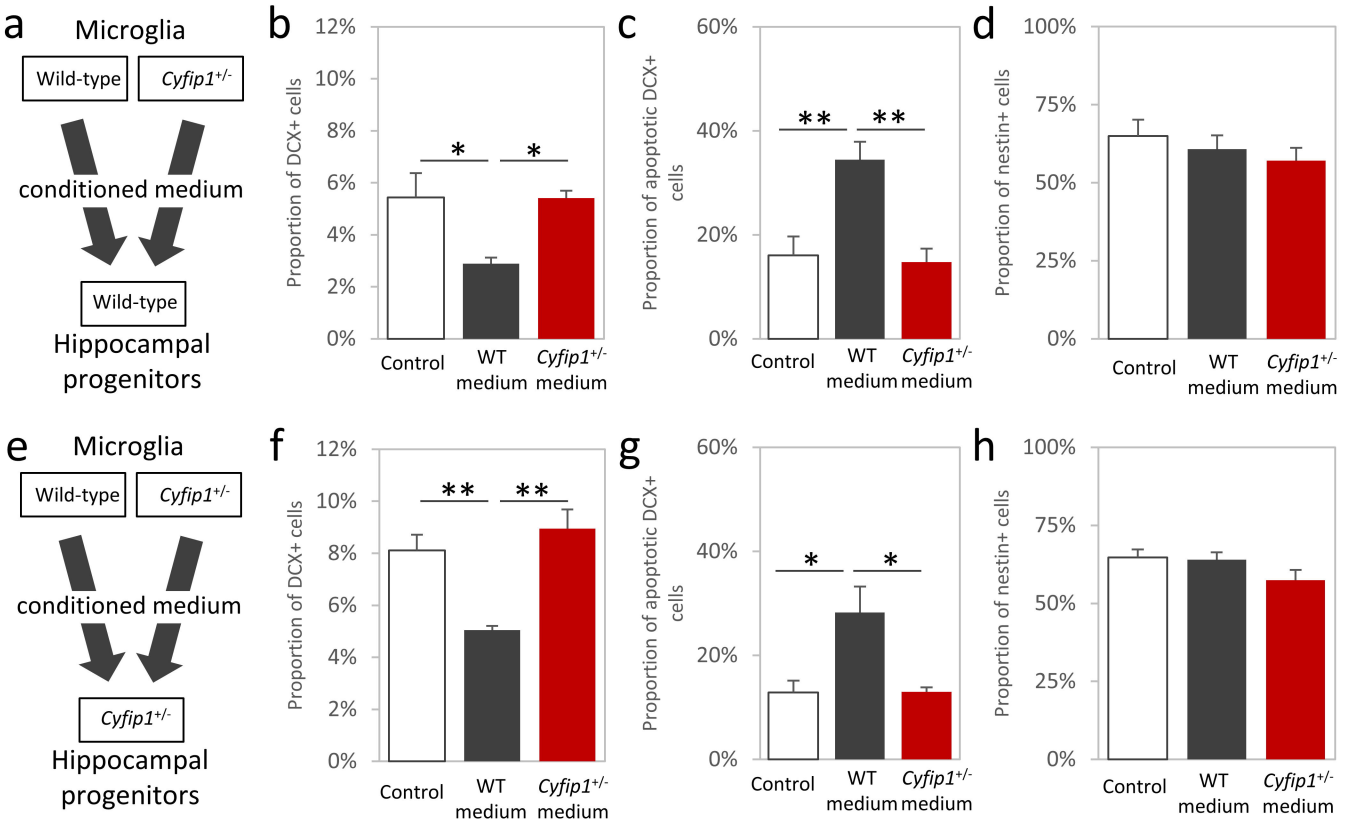

Iba1 / DAPI
a
Wt
Cyfip1+/-
b
Iba+ cells / mm²
250
200
150
100
50
0
Control
Cyfip1+/-
c
Fold change in Cyfip1 expression
20
15
10
5
0
***
Control
10 µg/ml LPS
d
Normalised CD68 levels
1.8
1.6
1.4
1.2
1
0.8
0.6
**
*
Control
10 µg/ml LPS
e
TNFα
IL6
IL10
CCL4
CCL5
CCL7
CCL12
CXCL1
CXCL2
CXCL10
Serpin E1
VEGF
Concentration (pg/ml)
***
Control
LPS
Wild-type
Cyfip1+/-

Figure 4


a
Microglia
Wild-type
Cyfip1+/-
conditioned medium
Wild-type
Hippocampal progenitors
b
Proportion of DCX+ cells
12%
10%
8%
6%
4%
2%
0%
*
*
Control
WT medium
Cyfip1+/- medium
c
Proportion of apoptotic DCX+ cells
60%
40%
20%
0%
**
**
Control
WT medium
Cyfip1+/- medium
d
Proportion of nestin+ cells
100%
75%
50%
25%
0%
Control
WT medium
Cyfip1+/- medium
e
Microglia
Wild-type
Cyfip1+/-
conditioned medium
Cyfip1+/-
Hippocampal progenitors
f
Proportion of DCX+ cells
12%
10%
8%
6%
4%
2%
0%
**
**
Control
WT medium
Cyfip1+/- medium
g
Proportion of apoptotic DCX+ cells
60%
40%
20%
0%
*
*
Control
WT medium
Cyfip1+/- medium
h
Proportion of nestin+ cells
100%
75%
50%
25%
0%
Control
WT medium
Cyfip1+/- medium

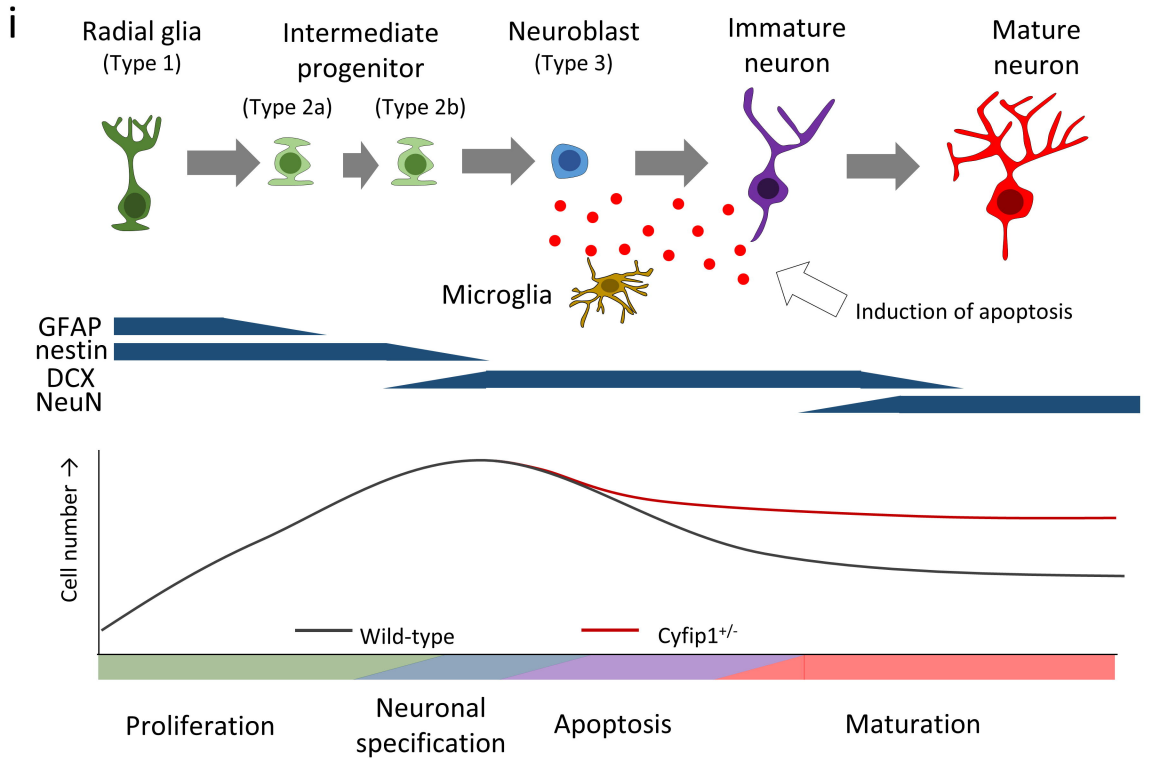

i
Radial glia
(Type 1)
Intermediate progenitor
(Type 2a)
(Type 2b)
Neuroblast
(Type 3)
Immature neuron
Mature neuron
Microglia
Induction of apoptosis
GFAP
nestin
DCX
NeuN
Cell number →
Wild-type
Cyfip1+/-
Proliferation
Neuronal specification
Apoptosis
Maturation

Figure 5

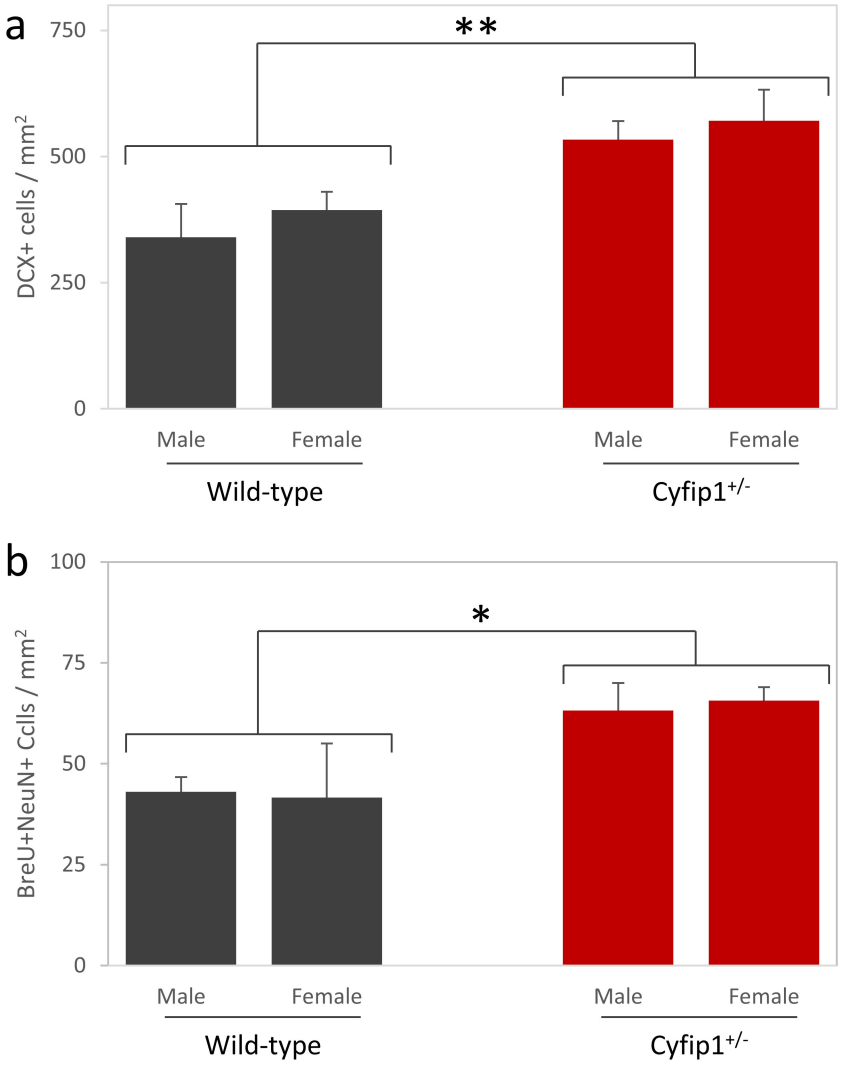
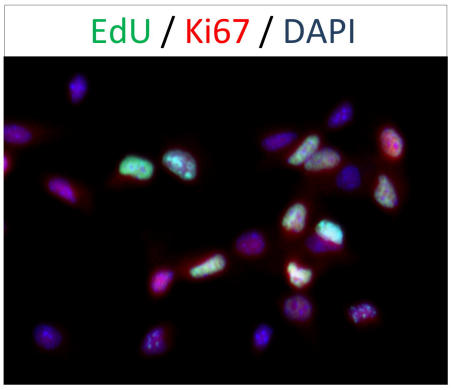

a
750
500
250
0
DCX+ cells / mm2
**
Male
Female
Male
Female
Wild-type
Cyfip1+/-
b
100
75
50
25
0
BreU+NeuN+ Cells / mm2
*
Male
Female
Male
Female
Wild-type
Cyfip1+/-

Figure S1


EdU / Ki67 / DAPI

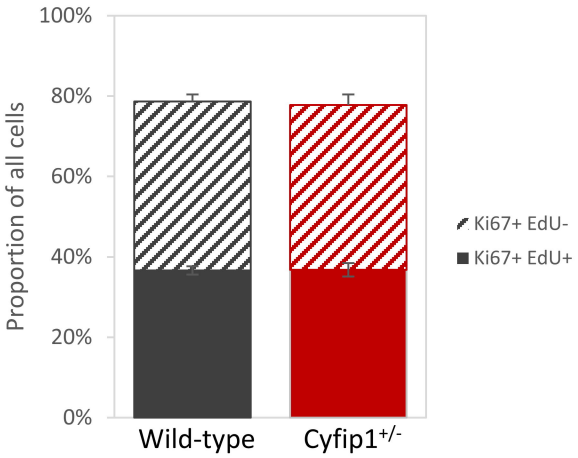

100%
80%
60%
40%
20%
0%
Proportion of all cells
Wild-type
Cyfip1+/-
Ki67+ EdU-
Ki67+ EdU+

Figure S2

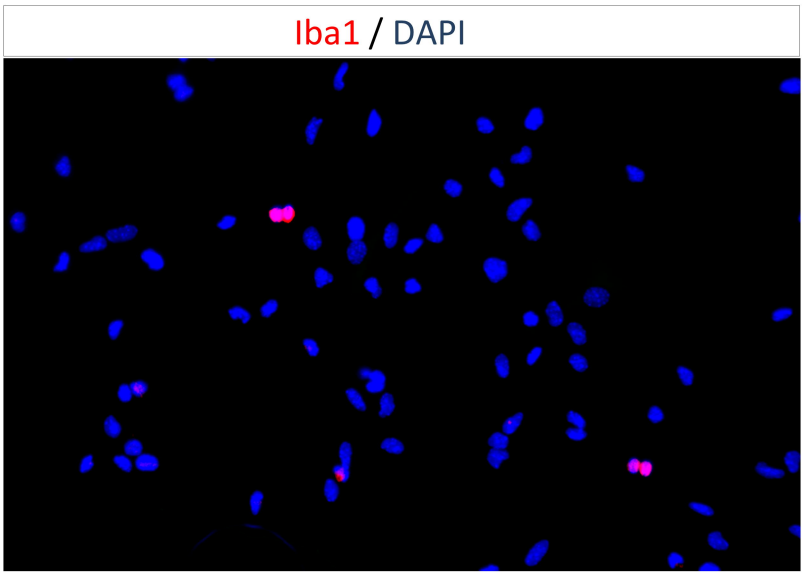


Figure S3

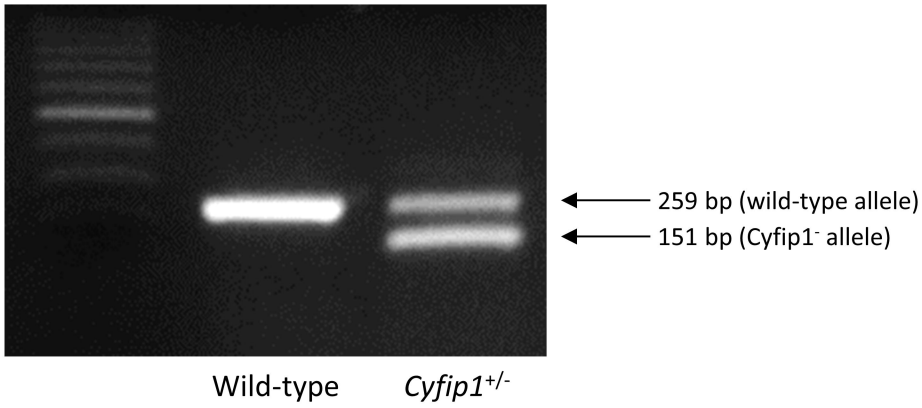

259 bp (wild-type allele)
151 bp (Cyfip1- allele)
Wild-type
Cyfip1+/-

Figure S4